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(71) Applicant: SOMAGENICS, INC. [US/US]; 239 Cypress Drive, Mountain View, CA 94043 (US).

(72) Inventors: JOHNSTON, Brian, H.; Somagenics, Inc., 239 Cypress Drive, Mountain View, CA 94043 (US). KAZAKOV, Sergei, A.; 3375 Alma Street #262, Palo Alto, CA 94306 (US). KISICH, Kevin, O.; 2451 Jonquil Court, Lafayette, CO 80026 (US).

(74) Agents: DREGER, Walter, H. et al.; Flehr Hohbach Test Albritton & Herbert LLP, Suite 3400, 4 Embarcadero Center, San Francisco, CA 94111–4187 (US).

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(57) Abstract

The present invention is directed to novel nucleic acid molecules and methods for their use. More specifically, the novel nucleic acid molecules of the present invention are capable of tightly and specifically interacting with a target molecule of interest not only through standard Watson–Crick base pairing, but also through additional fetaures which allow the antisense molecules to become topologically "locked" onto the target nucleic acid, thereby imparting improved transcription and translation inhibitory properties.

# ANTISENSE AND ANTIGENE THERAPEUTICS WITH IMPROVED BINDING PROPERTIES AND METHODS FOR THEIR USE

## FIELD OF THE INVENTION

The present invention relates generally to antisense and antigene oligonucleotides and their use as probes as well as diagnostic and therapeutic agents, and more particularly to antisense and antigene oligonucleotides which are capable of topologically linking to target nucleic acid molecules so as to impart tight binding characteristics and, in turn, improved translation and transcription inhibitory properties. The present invention also relates to novel methods for the platination of oligonucleotides to improve their antisense and triplex-forming properties and to allow those oligonucleotides to bind to double-stranded DNA through an antisense mechanism.

## BACKGROUND OF THE INVENTION

Antisense approaches to the therapeutic modulation of gene expression have been shown to be effective both in cultured cells and in whole animals (Crooke, *Antisense Nucleic Acid Drug Dev.* 8:115-122 (1998), Matteucci and Wagner, *Nature* 384:20-21 (1996) and Mesmaeker et al., *Acc. Chem. Res.* 28:366-374 (1995)). However, the standard antisense method, involving delivery of oligonucleotides modified for nuclease resistance, has several difficulties: Toxicity of phosphorothioate and other derivaties is a concern, cell-specific delivery is difficult, and levels with target cells

cells using novel antisense and antigene oligonucleotides that are capable of topologically linking to target nucleic acid molecules, thereby imparting tight binding properties and the ability to resist dissociation from the target. More particularly, the present invention is directed to a new generation of antisense and antigene agents for the specific control of gene expression. These agents bind to RNA and/or DNA target molecules not merely by the strength of Watson-Crick pairing (as do standard antisense agents), but employ additional features that "lock" the antisense or antigene molecule onto the target nucleic acid, thereby making it highly resistant to dissociation promoted by helicases, ribosomes or modifying enzymes. We herein demonstrate success in achieving extremely tight binding and have identified mechanisms responsible for this tight binding. Moreover, we herein establish that this method is very effective in blocking ribosome scanning in cell-free translation systems as well as in intact cells. This approach of using nucleic acid structural considerations to topologically "padlock" the antisense or antigene and target molecules together is a significant advance, for antisense and antigene therapy in general, for gene function analysis and target validation and for gene therapy, as a means for the controlled and cell-specific delivery of antisense and antigene molecules, in particular. Methods employing these antisense oligonucleotides both *in vitro* and *in vivo* as well as kits comprising them are also provided. Methods for the platination of oligonucleotides, for example, to improve their antisense and triplex-forming properties are also provided. In addition, we provide methods for detecting and amplification of nucleic acids based on mechanistic features of some of these antisense constructs.

## BRIEF DESCRIPTION OF THE DRAWINGS

**Figs. 1A-H. Exemplary Schemes for Employing Antisense Oligonucleotides Which Become Topologically Linked to the Target Molecule. A.** Presented is a general scheme showing a nucleic acid molecule topologically linked to a single-stranded target. The ball represents any of various ways for the ends of this molecule to interact following hybridization with the target with creation of at least one turn of helical interwinding. The third-strand interaction to form a triplex is optional. In one embodiment, the ball comprises a hairpin ribozyme moiety (see

formed between the murine TNFα mRNA target and the "folded" form of the ATR 1 antisense RNA (R1 or R4). **C.** Presented is the putative secondary structure of the complex formed between the TNFα mRNA target and the AT antisense RNA, containing only the antisense and triplex forming regions but lacking the hairpin ribozyme. **D.** Presented is the secondary structure and sequence of the essential core of HPR, showing domains referred to in the text. **E.** Autoradiograph of a 6% denaturing polyacrylamide gel showing RNA species produced by self-processing of primary HPR1 transcript. Lane 1, 5S RNA marker, lane 2, internally $^{32}$P-labeled RNAs showing all species, lane 3, RNAs labeled at their 3' termini by ligation to 5'-[$^{32}$P]pCp using T4 ligase. R2 and R3 are unprocessed and partially processed primary transcripts, whereas R1 is circular HPR and R4 is linear HPR. **F.** 6% denaturing polyacrylamide gel showing RNA species produced by self-processing of primary HPR1 transcript. Lane 1, gel purified circle, lane 2, gel purified linear, lanes 3 and 4, circle (R1) and linear HPR (R4), showing self-cleavage and self-ligation, respectively, after incubating with $Mg^{2+}$-containing buffer for 1 hour at 37°C.

**Fig. 3. Superiority of ATR 1 (Antisense-Triplex-Ribozyme) RNA over AT (Antisense-Triplex) RNA in Stability of the Complexes Formed with TNFα Target RNA.** Fig.3 shows an autoradiogram made after polyacrylamide gel electrophoresis of the gel purified linear ATR 1 RNA ("R4" form) in equilibrium with its circular form ("R1" form) (lanes 1-6) and AT RNA (lanes 7-12), after incubation alone (lanes 1, 4, 7 and 10) and either with 0.1 μg/μl (lanes 2, 5, 8 and 11) or 0.2 μg/μl (lanes 3, 6, 9, and 12) TNF1 RNA. All samples were mixed with equal volumes of 2xFLS (standard gel loading solution containing 90% formamide and 10 mM EDTA) and incubated either for 5 min. at 37°C (lanes 1-6) or for 2 min. at 95°C (lanes 7-12) before electrophoresis. "S" corresponds to the start location of the gel and "SC" refers to the location of the strongly interacting RNA complexes.

**Figs. 4A-B. TNFα-Luciferase Fusion Targets and Inhibition of Expression by ATR Antisense RNAs.** **A.** Presented is a schematic diagram and the nucleotide sequence of a DNA template for the TNFα-luciferase fusion gene. **B.** Presented is

**Fig. 9. Scheme For Using SELEX to Select Antisense Padlock Complexes That Can Be Locked to a Target by a Clasp Molecule. Top:** Presented is a scheme for selecting antisense complexes that can be locked to a target by a clasp molecule (grey ball). The target DNA, immobilized on a substrate, is mixed with a pool of DNA containing nucleotides randomized in the area shown in bold. **Bottom:** Example using c-myc as clasp and HER2 as target mRNA. n, sequences to be randomized. "xxx" represents CUU or ethylene glycol residues. In the triplex mode, x would be chosen to pair with the duplex formed by the other two strands, lending additional stability and permitting obstruction of translation. This scheme will adjust the lengths of the duplex and single-stranded linker regions so that there is poor binding without the protein clasp and good binding with it. In this process, the length of duplex required for binding by the truncated c-myc will automatically be selected.

**Fig. 10. Inhibition of TNFα Secretion by ATR Constructs.** Fig. 10 shows the inhibition of secretion of TNFα by RAW264.7 cells after treatment with control mRNA (m101; i.e., a padlock contruct with an irrelevant antisense region) or the various antisense constructs ATR 1, ATR 16a, ATR 16b or ALR 229.

**Fig. 11. Gel Shift Analysis on Denaturing Gels of ATR 1, ATR 16a, ATR 16b and ALR 229 Constructs.** Fig. 11 shows the results of gel shift analysis on denaturing gels of ATR 1, ATR 16a, ATR 16b and ALR 229 constructs. ATRs were incubated with $^{32}$P-labeled target TNFα RNA fragment ($^{32}$P-TT RNA). ATR 1 was used as a negative control because it possesses no antisense region corresponding to the target TNFα mRNA employed.

**Figs. 12A-D. Kinetics of Hybridization and Strong Complex Formation for ATR 1, ATR 16a, ATR 16b and ALR 229 Constructs with TNFα RNA.** Figs. 12A-D show the kinetics of hybridization and strong complex formation for the ATR 1, ATR 16a, ATR 16b and ALR 229 contructs with TNFα RNA target. cc, complementary complex; sc, strong complex.

platinum groups introduced into internal regions of antisense oligonucleotides through the modification of either sulfur atoms of internucleotide phosphorothioates or N7-positions of purine bases. **b-e,** Cationic platinum groups attached to antisense oligonucleotide constructs through non-hybridizing sequences or linkers by modification of sulfur atoms of internucleotide (and/or terminal) phosphorothioates, or thio-pyrimidines, or thio-containing peptides or other organic oligomers. • - Represents platinum group attached to oligonucleotide prior to hybridization. Each pair of diagrams shows nucleic acid complexes made by both unmodified and platinated oligonucleotides.

**Figs. 19A-E. Platination patterns for oligonucleotides to stabilize triple-helical complexes with either DNA duplexes, or hairpin and single-stranded RNAs. a,** "Classic" triplexes with either homopurine or homopyrimidine tfos. **b,** alternate strand triplexes with oligonucleotides containing both homopurine and homopyrimidine triplex-forming domains. **c,** triplexes formed by hybrid oligonucleotides containing two triplex-forming domains connected by a non-hybridizing linker. **d,** oligonucleotides forming both a duplex and a triplex with a target structure containing an internal loop. **e,** triplex "clamps" featuring hairpin-like structure. • – represents a platinum group attached to oligonucleotide prior to hybridization. Each pair of diagrams shows nucleic acid complexes made by both unmodified and platinated oligonucleotides.

**Fig. 20. Cationic platinum derivatives of antisense oligonucleotide constructs designed to open and bind double-stranded regions of DNA and RNA by substituting for competing complementary sequences.** An intramolecular self-complementary structures of the antisense oligonucleotides are destabilized by strong ionic repulsion and steric hindrance of the platinum groups. • - Represents platinum group attached to oligonucleotide prior to hybridization. Each pair of diagrams shows nucleic acid complexes made by both unmodified and platinated oligonucleotides.

**Fig. 21. Diethylenetriamine catalyzes the platination of oligonucleotides.** Autoradiogram, after electrophoresis on 20 % denaturing polyacrylamide gel, of [5'-

oligonucleotide and of the products of its modification by (30 $\mu$M $K_2PtCl_4$ + 1 mM dien) in 10xTAE buffer for 2 h at 45° C. Concentrations of added non-radioactive oligonucleotide and the corresponding platinum:oligonucleotide molar ratios were as shown. No $K_2PtCl_4$ was added to samples 1-2.

**Fig. 25. Schematic Representation of Binary Recombinant RNA (replicase) Probe Hybridized to the HIV-1 pol RNA Target.** The replication probe consists of approximately one-half of the MDV-1 (+) RNA (the template for Qβ replicase when whole) joined at the small arrows to a 12 nt sequence complementary to the target, then one-half of the hairpin ribozyme substrate sequence (7 to 10 nt), and terminating at the ligation site. These replication probes cannot be amplified unless they are hybridized to their target and ligated to the hairpin ribozyme catalytic core (not shown), which is itself folded into the active conformation only in the presence of target. Upon ligation (at the site shown by the arrowhead) the complete molecule is replicated by Qβ replicase in a process that detaches it from the target and ribozyme and may result in its folding into the structure shown on the right. The 40-nt inserted sequence containing the ligation site for the hairpin ribozyme is shown above the small arrows.

**Fig. 26. Scheme for Ribozyme-Assisted RNA Amplification Using Qβ Replicase.** Presented is a schematic representation of the overall scheme for amplifying nucleic acids using a ribozyme-assisted approach and Qβ replicase.

**Fig. 27. Schematic Representation of the Recombinant RNA Capture Probe Bound to a Complementary Sequence of HIV-1 RNA Target.** Presented is a schematic representation of the recombinant RNA capture probe (total 60 nt in length) bound to a complementary sequence of HIV-1 RNA target (nt 4577-4760). The 12 nt substrate sequence for the target-dependent hairpin ribozyme (cleavage site shown by arrows) is attached both to the 45-nt hybridization probe through the oligo-U bridge from its 3'-end and to the magnetic bead from the 5'-end. the extended U-bridge is to permit ease of docking with target-bound domain E.

which ATR1 is an example. H. Presented is the molecule shown in G before covalent closure.

**Fig. 31. Scheme for Using Hammerhead Ribozymes to Detect any Molecule.** Fig. 31 presents a scheme for using a hammerhead ribozyme to detect the presence of any molecule (filled oval), which upon encountering the probe, assembles an optomer from dangling ends to which it specifically binds, thereby stabilizing the active conformation of a ribozyme. Subsequent cleavage detected by any of several techniques such as release of biotin from a solid support.

**Fig. 32. Scheme for Using Hammerhead Ribozymes to Detect any Nucleic Acid Sequence.** Fig.32 presents a scheme for using a hammerhead ribozyme to detect the presence of any nucleic acid sequence. Cleavage indicates a signaling event such as fluorescence or stimulation of an enzyme.

## DETAILED DESCRIPTION OF THE INVENTION

The present invention is directed to improved antisense and antigene oligonucleotide compositions and methods for down-regulating the expression of various proteins in cells using novel antisense and antigene oligonucleotides which are capable of resisting dissociation from target nucleic acid molecules. More particularly, the present invention is directed to novel antisense and antigene molecules and methods of their use, wherein the novel antisense and antigene molecules are capable of tightly binding to a target nucleic acid not only through standard Watson-Crick pairing (as do standard antisense agents), but also employ additional features that topologically "lock" the antisense or antigene molecule onto the target nucleic acid molecule, thereby making it highly resistant to dissociation promoted by helicases, ribosomes or modifying enzymes and, in turn, imparting improved translation inhibitory properties.

By "topologically linked" is meant that the antisense or antigene oligonucleotide circularizes around the target molecule. For example, if an initially linear antisense or antigene molecule binds to an mRNA target, wraps around it, and then circularizes, it would be very difficult to displace. Unless an endonucleolytic

antisense or antigene molecules of the present invention is the hairpin ribozyme which is derived from the minus strand of the satellite RNA associated with tobacco ringspot virus (Buzayan et al., *Nature* 323:349-353 (1986a), Feldstein et al., *Gene* 82:51-63 (1989) and Hampel and Tritz, *Biochemistry* 28:4929-4933 (1989)). The catalytic domain of the hairpin ribozyme has a compact and stable structure and is capable of autocatalytically cleaving and ligating at a specific site to interconvert between a covalently closed circle and a non-covalently closed form which possesses a 5'OH group and a 2',3'-cyclophosphate terminus (Fig. 2). As such, when a standard antisense or antigene RNA molecule (i.e., one which generally binds to a target only through standard Watson-Crick base pairing) is modified to contain the catalytic domain of the hairpin ribozyme, the modified antisense RNA is not only capable of recognizing and binding to its target through standard Watson-Crick base pairing and other similar interactions, but also is capable of becoming "locked" onto the target molecule through the catalytic function of the ribozyme. The ribozyme may be catalytically active when the antisense molecule (or nucleic acid encoding it) is introduced into the cell, or may be inactive when introduced and may become activated upon subsequent events which will be described below. Various catalytic RNA molecules are known in the art and may be routinely employed for linkage to an antisense oligonucleotide to facilitate topological linkage to a target nucleic acid. Examples of antisense or antigene constructs that comprise a catalytic RNA are the ATR constructs described below (see Figs. 2 and 7).

To further stabilize the antisense or antigene molecule/target complex, one can also introduce a triplex-forming region into the antisense or antigene molecule. A triplex-forming region is a nucleic acid sequence which is incorporated into the antisense or antigene molecule and which functions to form a triplex with the duplex that is created between the complementary sequences of the antisense or antigene molecule and its target. While more detail regarding triplex formation and the sequences required therefor is presented below, it is evident to those skilled in the art that the ability to employ triplex-forming sequences will depend upon the sequence of the target nucleic acid and the corresponding antisense or antigene molecule.

cytoplasmic extracts from HeLa cells, circular ribozyme molecules have better resistance to nuclease degradation than do linear forms of the ribozyme (Puttaraju et al., *Nucl. Acids Res.* 21:4253-4258 (1993)).

Another advantage to circularly linking an antisense or antigene oligonucleotide to a target nucleic acid molecule is improved strength and specificity of binding as compared to that obtained with linear antisense or antigene oligonucleotides. It has been shown that antisense circles as small as 30 to 40 nucleotides can form regular 15-18 bp duplexes with target sequences in mRNAs (Dolinnaya et al., *Nucl. Acids Res.* 21:5403-5407 (1993)). In the case of DNA, circular oligodeoxyribonucleotides have been shown to bind effectively to single-stranded homopurine or homopyrimidine nucleic acids by triplex formation (Kool, *J. Amer. Chem. Soc.* 113:6265-6266 (1991)). They show higher sequence selectivity and less tolerance of mismatches with target sequences than do ordinary linear oligomers (Kool (1991), *supra*, Prakash and Kool, *J. Chem. Soc. Chem. Commun.* 1161-1163 (1991), Prakash and Kool, *J. Am. Chem. Soc.* 114:3523-3527 (1992), Wang and Kool, *Nucl. Acid. Res.* 22:2326-2333 (1994)). A comparison of circular RNA and DNA oligonucleotides of the same sequence showed that RNA circles bind single-stranded RNAs with considerably higher affinity than do DNA circles, even without topological linkage of the oligonucleotides to the targets as described herein (Wang and Kool (1994), *supra*).

Small RNA molecules are also advantageous as antisense and antigene agents because they minimize possibilities for folding into alternate conformations that can interfere with target recognition (Forster and Symons, *Cell* 50:9-16 (1987), Hélène and Toulmé, *Biochim. Biophys. Acta* 1049:99-125 (19900 and Dolinnaya et al. (1993), *supra*). However, for *in situ* expression, additional sequences are needed for high-levels or cell-type-specific expression. If a circular RNA is to be the active antisense or antigene agent for the reasons described above, the best way to lessen conformational problems is to autocatalytically excise from the final circle any sequences that are irrelevant for target binding.

We consider herein that the hairpin ribozyme is appropriate for topologically linking an antisense or antigene oligonucleotide to a target nucleic acid because of the compact and stable structure of its catalytic domain (Feldstein and Bruening,

purine third-strand motif to permit triplex formation at sequences other than pure homopurine-homopyrimidine blocks. This capability will permit the antisense- or antigene-triplex approach proposed herein to be applicable to a greater variety of target sequences than would otherwise have been possible.

Triplex formation may require protonation of cytosines in the third strand. Because the $pK_A$ of cytosine is shifted substantially toward neutral in a triplex, the triplexes proposed are expected to be stable under physiological conditions. However, if triplex stability is limiting, constructs will be synthesized with 5-methyl cytosine in place of cytosine at the third strand positions, which shifts the pK to higher values.

Any gene in which at least a portion of the coding and/or non-coding sequence is known or readily obtainable and which would benefit from a lower expression thereof will serve as targets for the novel antisense and antigene molecules of the present invention. As will be apparent from the present disclosure, the antisense and antigene oligonucleotides of the present invention can be routinely adapted to bind to and become topologically linked to virtually any target nucleic acid molecule of interest. As such, the presently described antisense molecules represent a major step forward in the field of antisense therapeutics.

**Delivery into Cells:** Previous studies have demonstrated that catalytic RNA can be delivered to cells utilizing cationic lipids, such as N[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethylammonium chloride (DOTMA) (Sioud et al., *J. Mol. Biol.* 242:831-835 (1991)). Other investigators (Zhu et al., *Science* 261:209-211 (1993)) have used a 1:1 ratio of DOTMA with dioleoylphosphatidylethanolamine (DOPE) to achieve systemic expression of plasmid DNA following intravenous injection of plasmid DNA:DOTMA:DOPE complex into mice. Other cationic lipid formulations have become commercially available (e.g. DOSPA:DOPE, DOTAP, DMRIE:cholesterol, DDAB:DOPE, and others) and offer improved expression of DNA. A 1:1 mixture of dioctadecylamidoglycylspermine (DOGS):DOPE is effective to introduce ribozyme constructs into cells both *in vivo* and *in vivo* (see, e.g., Kisich and Erickson, *J. Leukocyte Biol. Suppl.* 2:70 (abstract) (1991a) and Kisich and Erickson, *FASEB J.* 4:1860 (abstract) (1991b)). As presented below, 1:1

and Van Dyke, 1995; Pallan and Ganesh, 1996) favor triplex (both pyrimidine- and purine-rich) formation.

However, the inhibitory effect of $K^+$ can be completely overcome or reversed by physiological concentrations of such favorable cofactors as $Mg^{2+}$, spermine$^{4+}$ or spermidine$^{3+}$ (Musso and Van Dyke, 1995; Olivas and Maher, 1995a). Approaches to destabilizing aggregates of purine-rich TFOs under physiological conditions would aid their biological applications (Olivas and Maher, 1995a; Svinarchuk et al., 1996). A remarkable solution for this problem has been accomplished by using 6-thioguanine substituted for guanine, presumably because the increased radius and decreased H-bonding ability of sulfur in the C6-position destabilize potential guanine tetraplexes (Olivas and Maher, 1995b). We propose that platination of the guanine N7-position (see Fig. 17B), which also plays a key role in tetraplex formation through H-bonding, could result in a similar suppressing effect on guanine quartet-mediated aggregation of TFOs.

**Attachment of functional groups to triplex-forming and antisense oligonucleotides.** The enhancement of triplex stability by cations can be further exploited by their conjugation with the TFOs. For example, the attachment of different cationic peptides (Tung et al., 1996) and polyamines such as spermine (Tung et al., 1993) to the 5'-end of the homopyrimidine oligonucleotides boosts the stability of triplexes while having no affect on the stability of the underlying double helix.

Here, we propose the attachment of conjugates of platinum and polyamine cations to TFOs. Currently, there is no information available about the effect on triplex stability of the of platinum complexes tethered to TFOs. However, it is reasonable to expect that appropriate "platination" of oligonucleotides will not compromise their ability to form triplexes, and might even have positive effects. For example, some other metal complexes, [$Fe^{2+}$-EDTA] and [$Cu^+$-phenanthroline], attached to oligonucleotides at either their termini, bases or sugar moieties, have been successfully used as chemical probes of triplex structures (Moser and Dervan, 1987; Francois et al., 1989; Beal and Dervan, 1992; Jayasena and Johnston, 1992; Thuong and Helene, 1993; Shimizu et al., 1994; Tsukahara et al., 1996).

**Specific platinum labeling of phosphorothioate nucleic acids.** Platinum (and mercury) compounds can specifically bind to sulfur atoms either occurring naturally (e.g., in tRNA molecules) or synthetically incorporated in nucleic acids (Pal et al., 1972; Jones et al., 1973; Scheit and Faerber, 1973; Strothkamp and Lippard, 1976; Strothkamp et al., 1978; Szalda et al., 1979; Chu and Orgel, 1989, 1990, 1991, 1992; Elmroth and Lippard, 1994; Slavin et al., 1994). The resulting modified nucleic acids are potentially useful for X-ray crystallography, electron microscopy or other applications requiring heavy metal labeling (Strothkamp and Lippard, 1976; Lippard, 1978; Strothkamp et al., 1978; Szalda et al., 1979), as well as antisense and antigene probes (Chu and Orgel, 1989, 1990, 1991, 1992).

However, little quantitative information is available about the reactivity of phosphorothioates in nucleic acids toward platinum reagents, and only a few kinds of such reagents have been studied so far. One of them is $[(terpy)Pt^{II}X]^{n+}$ (Fig. 15), which has demonstrated almost quantitative binding to the sulfur atoms in nucleoside monophosphorothioates (AMPS and UMPS) and double-stranded poly($_S$A-U) (Strothkamp and Lippard, 1976) as well as in yeast $tRNA^{Phe}$ containing a modified $C_S$-$C_S$-A at the 3'-end (Szalda et al., 1979). Reactions between 4-40 $\mu$M of this platinum reagent with $r_f$ (platinum to nucleic acid molar ratio) in the range 0.005 to 5 were performed in [50 mM Tris-HCl (pH 7.5), 0.1 M NaCl] buffer at 25° C for 10 min. Cation exchange column chromatography on AG50W-X8 (Bio-Rad) was successfully used to remove all noncovalently bound $[terpy)PtCl]^+$. There was no evidence for loss of platinum from, or degradation of, phosphorothioate linkages in the purified platinated polyribonucleotides. The data showed that the platinum reagent binds selectively to the phosphorothioate groups in these polyribonucleotides even if the platinum reagent was in excess since no platinum binding to corresponding all-phosphodiester RNA was found under the same conditions (Strothkamp and Lippard, 1976). Binding to the $C_S$-$C_S$-A-modified tRNA molecule was complete with the attachment of two platinum complexes, one for each phosphorothioate group. Even extended incubation periods of up to 24 h resulted in no additional binding of [(terpy)Pt] moiety per the tRNA molecule at the $1 > r_f > 5$ (Szalda et al., 1979).

into phosphorothioate oligonucleotides by $K_2[PtCl_4]$ having four reactive Pt-Cl coordinates, noticing that such crosslinking products (if they were formed) were not stable and underwent further chemical transformation (Chu and Orgel, 1990a).

**Diethylenetriamine Catalyzes Platination of Oligonucleotides.** In aqueous solutions, $[PtCl_4]^{2-}$ (Fig. 15) is known to react very slowly with either polynucleotides alone (Wherland et al., 1973; Chu and Orgel, 1989; Kasianenko et al., 1995) or diethylenetriamine (dien) alone (Fig. 16) (Watt and Cude, 1968; Mahal and Van Eldik, 1987), forming complex mixtures of products in both cases. We found, that in the three-component mixtures, oligonucleotide platination in the presence of dien proceeds rapidly (<2 h at 45° C) and with a high yield of homogeneous products even at low, micromolar platinum concentrations (10-30 $\mu$M). To prepare 50-100 pmoles of the platinum oligonucleotide derivatives, only 0.3 nmoles of the platinum reagent is required. Positively charged dien$H_2$2+ presumably counteracts the electrostatic repulsion between $[PtCl_4]^{2-}$ and polyanionic phosphate backbone, bringing these two together in very close proximity and stimulating initial platinum binding to oligonucleotide (Fig. 16). Subsequently, the oligonucleotide accelerates chelation of the tethered platinum by dien due to preassociation of the cationic polyamine with the negatively charged nucleic acid surface. The final reaction products presumably consist of diethylenetriaminoplatinum(II), $[dienPt]^{2+}$, forming chemically inert and thermodynamically stable adducts through sulfur of POS (Fig. 17A) or the N7 of guanine residues (Fig. 17B). These adducts are compact and carry a positive charge with potential to promote hybridization to other nucleic acids. (Lepre and Lippard, 1990). The additional positive charge of the platinum groups also allows easy separation of platinated oligonucleotides from reaction mixtures by either preparative electrophoresis (as used in this work) or HPLC. We also consider metallo-affinity chromatography as an alternative, one-step method of purification of the platinated phosphorothioate oligonucleotides.

The major advantages of this new synthetic method are related to the labeling of oligonucleotide probes by radioactive molecules. This is a one-tube reaction which can be performed in any biochemical lab. No special equipment or skills for multiple steps, radioactive synthesis of platinum compounds and product isolation

biomedical, biochemical and biophysical applications such as antisense and antigene technology, heavy atom labeling of nucleic acids for electron microscopy, metallo-affinity chromatography of nucleic acids, catalytic RNA (ribozymes), enzyme biochemistry, nucleic acid-protein interactions, and oligonucleotide-directed mutagenesis.

The fact that phosphorothioate oligonucleotides have already been subjected through extensive biological tests would make it easy to repeat similar studies using the platinated analogs.

**Preparation and Purification Methods of Phosphorothioates.** A terminal phosphorothioate can be easily attached to the 5'-end of both RNA and DNA of unmodified oligonucleotides by polynucleotide kinase and ATPγS, and the 3'-end of RNA (but not DNA) can be phosphorothioated by RNA ligase and dpCp(S) (Eckstein, 1985).

The non-bridging phosphorothioates can be incorporated into the backbone of nucleic acids by chemical or enzymatic methods (Zon and Stec, 1991). Effective analytical and preparative chromatography (reverse phase HPLC) methods for purification and analysis of phosphorothioate oligonucleotides were developed for their clinical evaluation as antisense agents (Zon and Geiser, 1991; Zon and Stec, 1991; Padmapriya et al., 1994; Gerstner et al., 1995). Due to the present unavailability of a chemical stereo-specific synthesis, synthetic oligonucleotides with $n$ phosphorothioate residues are mixtures of $2^n$ possible diastereisomers ($R_p$ and $S_p$). Only the stereoisomers of oligonucleotides with a few phosphorothioate residues can be separated and purified by HPLC (Chu and Orgel, 1990). Enzymatic synthesis of phosphorothioate polynucleotides (RNA or DNA) using appropriate templates, polymerases, and thiotriphosphate nucleotides yields stereo-specifically the $R_p$ isomers. The purification protocols of phosphorothioate polynucleotides longer than 50-mers are usually based on metal-affinity, chromatography, and electrophoresis (see below).

**Phosphorothioate physico-chemical properties.** Replacing an oxygen by sulfur in a phosphate reduces the charge on the remaining oxygens while increasing the negative charge on sulfur. Protonation of phosphorothioates occurs preferentially on oxygen rather than sulfur since phosphorothioates are stronger acids (have lower

Dyke, 1995; Joseph et al., 1997; Lacoste et al., 1997) or even provided a modest increase in the stability (Latimer et al., 1989; Hacia et al., 1994; Musso and Van Dyke, 1995) depending on their sequences. In contrast, all-POS homopyrimidine (Kim et al., 1992; Hacia et al., 1994) and GT-containing oligonucleotides (Lacoste et al., 1997) bind target duplex DNA with drastically reduced affinities in comparison with relative all-phosphodiester oligonucleotides (Kim et al., 1992; Hacia et al., 1994). In general, the binding energy of triplex formation for derivatives of pyrimidine TFOs decreased with the number of POS linkages (Lacoste et al., 1997). However, pyrimidine TFOs containing up to 20% POS linkages can repress a transcription with efficiency comparable to that of all-phosphodiester oligonucleotides (Alumni-Fabbroni et al., 1994).

In fact, because they have high resistance towards exonucleases, oligodeoxynucleotides with POS-capped ends should be superior to normal oligonucleotides for experiments in vivo (Alumni-Fabbroni et al., 1994).

Relatively new bifunctional oligonucleotide probes, combining antisense and triplex-forming domains (see Fig. 19D and E), allow specific targeting of single-stranded and hairpin regions in mRNAs (Brosalina et al., 1993; Kandimalla et al., 1995; Francois and Helene, 1995; Moses and Schepartz, 1996). Such TFOs, as well as oligonucleotides recognizing DNA by alternate strand triple helix formation (Beal and Dervan, 1992; Jayasena and Johnston, 1992) and DNA's containing two TFO domains connected by a flexible linker, (Kessler et al., 1993) have convenient sites between these domains for introducing POS linkages or reactive, nonhybridizing nucleotide sequences (see Fig. 19B and 19C). We believe these sites seem to be most appropriate for chemical post-modification (e.g., by platinum reagents) without damaging the ability of these oligonucleotides to form specific complexes with nucleic acids targets. Recently, it was shown that the phosphorothioate internucleotide linkages inside a loop of the parallel-stranded hairpin complexes (see Fig. 19E) do not affect the stability of the triple helix complexes (Tsukahara et al., 1997). However, no other information is currently available on this topic.

**Reactivity of the phosphorothioates versus purines in nucleic acids.** Although both the N7- and N1-positions of adenosine are known to bind platinum, Strothkamp

more reactive aquoplatinum complex before interaction with nucleotides ( the major pathway):

$$[(dien)PtCl]^+ + H_2O \text{ ----> } [(dien)Pt(H_2O)]^{2+}$$

$$[(dien)Pt(H_2O)]^{2+} + NMP \text{ ---> } [(dien)Pt(NMP)]$$

The magnitude of the second-order rate constant for thionucleotides is about 20-fold greater than that for GMP and about 50-fold greater than for AMP (Slavin et al. 1994).

Since sulfur reacts with the [Pt-amine] compounds mainly through the directly substitution of the $Cl^-$ ligand without prior aquotation, the reaction between $[(dien)PtCl]^+$ and glutathione (HSDR) is nearly independent of the $[Cl^-]$ concentration whereas the reaction with GMP can be completely inhibited at high concentration of NaCl (Reedijk, 1991). In contrast, $[(dien)Pt(H_20)]^{2+}$ will almost selectively react with GMP (Reedijk, 1991).

Elmroth and Lippard (1994) showed that the rate of platination of GG site (through N7) in d(TTTTTTTTGGTTTTTTTT) by cis-$[Pt(NH_3)(NH_2C_6H_{11})Cl(H_20)]^+$ is approximately 35-fold higher than that in the dinucleotide d(GG), and only about 3-fold less reactive than the phosphorothioate site in d($TTTTTTT_sTTTTTTT$), irrespective of whether the oligonucleotide was single- or double-stranded oligonucleotide structure.

In the reaction with platinum reagents, $G_n$ clusters (where $n^3 2$) are the most reactive sites in DNA (Bruhn et al., 1990; Lepre and Lippard, 1990; Gonnet et al., 1996). At GGG (and longer G tracts), the N7 of the central residue is the most nucleophilic site(s), and $[(dien)PtCl]^+$ preferentially attacks this site (Yohannes et al., 1993).

Theoretically, if one $[L_3Pt]^{2+}$ group (where L is an amino ligand) is already attached to a guanine, it is expected to repel a second one reacting with an adjacent guanine. However in model experiments using $[(NH_3)_3PtCl]^+$ (Fig. 15), such repulsive effect of the first $[(NH_3)_3Pt]^{2+}$ group tethered to a guanine was found to be very moderate and did not prevent the platination of the second guanine in d(CTGGCTCA) even under stoicheometric conditions (Reeder et al., 1996). This result opens the possibility of a multiple platination of adjacent guanines in $G_n$ clusters.

Chimeric RNAs consisting of the minimal hairpin ribozyme sequence plus antisense and triplex-forming moieties (ATR 1) targeted to the intended target sequence of TNFα mRNA were designed as shown schematically in Figure 2A. A 150-bp DNA fragment encoding the T7 promotor, a 21-nt sequence complementary to a pre-selected region of TNFα RNA ("A"), a potential triplex-forming sequence ("T"), and the sequence of the minimal hairpin ribozyme was assembled from four overlapping oligonucleotides using T4 DNA ligase, amplified by the polymerase chain reaction (PCR), and transcribed by T7 RNA polymerase to generate the precursor (pre-ATR 1) RNA. Control experiments used an RNA species designated "AT" which possessed the antisense and triplex forming sequences required for forming a complex with the TNFα RNA target but which lacks the catalytic hairpin ribozyme domain.

Self-processing at 37°C of primary pre-ATR 1 RNA during transcription at the sites flanking the mature ATR 1 sequence (see "cleavage/ligation" sites in Figure 2A) resulted in the production of a number of RNA species (designated R2, R3a, R3b and R4) (Fig. 2E). By comparison of the length and analyzing the end structures of these individual gel-purified RNA species and the ways by which they can interconvert, we have unequivocally identified them as unprocessed transcript (R2), semi-processed linear ATR 1 (R3a and R3b), fully processed linear ATR 1 RNA (R4), and the circular form of mature ATR 1 RNA (not shown). All these RNA processing events are the result of autocatalytic cleavage by the internal ribozyme moiety. The identification of these RNA species was supported by 5'- and 3'-end labeling experiments (data nor shown). We have found that the interconversion of the linear R4 and circular species by self-ligation and self-cleavage (Fig. 2F) occurs under a wide range of conditions, which is in agreement with data obtained for other hairpin "miniribozymes" (Buzayan et al. (1986), *supra*, Hampel and Tritz (1989), *supra*, Chowrira et al., *Biochemistry* 32:1088-1095 (1993) and Feldstein and Bruening (1993), *supra*).

Figures 2B and 2C show the putative secondary structures of the complexes formed between the TNFα RNA target and the ATR 1 and AT antisense RNA species. Specifically, in the design of the ATR 1 structure, we incorporated a triplex-forming element in order to bring the ends of the hairpin ribozyme domain

min. at 37°C. All samples were mixed with equal volumes of 2xFLS (standard gel loading solution containing 90% formamide and 10 mM EDTA) and incubated either for 5 min. at 37°C (lanes 1-6 of Figure 3) or for 2 min. at 95°C (lanes 7-12 of Figure 3) before electrophoresis.

We found that ATR 1 antisense RNAs could form ultrastrong complexes with the target TNFα RNA molecule. These complexes appeared to be stable enough to be detected as several individual bands with close mobility when examined by denaturing electrophoresis in 6-8% denaturing polyacrylamide gels containing 8M urea, 2 mM EDTA at 45°C (see Figure 3, lanes 2-3). These findings agree with data reported on retardation of highly structured RNAs in denaturing polyacrylamide gels (Dante et al., *Anal. Biochem.* 225:348-351 (1995)). Moreover, the electrophoretic mobility of complexes between the [$^{32}$P]-labeled chimeric RNAs and nonradioactive TNF1 RNA was slightly retarded compared to that of [$^{32}$P]-labeled TNF1 alone.

In control experiments we used the AT RNA which, as described above, lacks the hairpin ribozyme domain but retains the sequences capable of forming the specific complex(es) with the TNF1 RNA (see Figure 2C), as well as "m101", a control RNA containing the minimal hairpin ribozyme domain plus an irrelevant sequence in place of the antisense and triplex forming sequences (see Fig. 2D). Complexes formed between [$^{32}$P]-labeled AT RNA and non-radioactive TNF1 under the optimum conditions dissociate during electrophoresis, producing a smear behind the principal band of AT RNA (Figure 3, lanes 5-6). Similar smearing has been reported for the gel-electrophoresis analysis of complexes formed between the hairpin ribozyme and its substrate analogs that are additionally stabilized by a long intermolecular duplex (Feldstein et al., *Proc. Natl. Acad. Sci. USA* 87:2623-2627 (1990)). No specific retardation or smearing was detected during similar gel-electrophoretic analysis of mixtures of [$^{32}$P]-labeled m101 and 0.02–0.2 μg/μl TNF1 RNAs (data not shown).

When the RNA complexes were dissolved in 45% formamide and 5 mM EDTA, heated at 95°C for 2 min. and subjected to polyacrylamide gel electrophoresis under denaturing conditions, only a small amount of the ultrastrong complexes between TNF1 and [$^{32}$P]-labeled ATR 1 RNA was detected, and all less stable complexes

of either ATR 1 or AT antisense RNA for 1 hr at 37°C. The resulting sense-antisense complexes were then added to a rabbit reticulocyte lysate (Promega, Madison, Wisconsin). Following *in vitro* translation of the complexes, luciferase production was measured. A T7 promoter-luciferase expression plasmid (designated herein as "PS") lacking the TNFα target sequence was used as a further control.

The results of these experiments are presented in Fig. 4B. Specifically, the results in Fig. 4B demonstrate that ATR 1 antisense RNA is much more effective at inhibiting translation of PTS mRNA than is the AT antisense RNA. For an optimal [ATR1:target] molar ratio of 30:1, incubation of ATR 1 antisense RNA with PTS mRNA caused $\geq$ 95% inhibition of its translation, whereas hybridization with PS lacking the TNFα sequence resulted in almost no suppression of translation. We assume that the slight sequence non-specific inhibition of control PS mRNA is due to the chance occurrence of short regions of complementarity between these RNAs that could lead to the formation of complexes under the conditions of the translation reaction. Such observations are common for the RNA-RNA antisense approach (Eguchi et al., *Ann. Rev. Biochem.* 60:631-652 (1991)) and could be reduced by intracellular "proof-reading" protein cofactors.

**D. Other Anti-TNF ATRs**

The ATR 16a, ATR 16b and ALR 229 constructs (see Fig.7) are similar to the ATR 1 construct except that they are directed to different regions of the TNFα target molecule. ATR 16a differs from ATR 1 in that is is targeted to a different homopurine sequence, which is located in the 5' UTR of the TNF RNA, it has a shorter triplex-forming sequence (to ensure that there would be more turns of duplex rather than triplex in the complex) and the triplex forming sequence was proximal to the ribozyme ligation site, wherein in ATR 1 it is distal. ATR 16b is identical to ATR 16a except that the linker connecting the triplex-forming region and the helix adjacent to the ligation site is longer. ALR 229 contains no triplex-forming region, but instead an $(AAC)_6$ loop (a sequence chosen to provide some self-stacking but no self-pairing structure). Hence, ALR 229 is not restricted to targeting triplex-forming homopurine sequences but, like ATR 1, is targeted to a coding region.

denaturing gel. For ATR 16a, ATR 16b and ALR 229, the inital kinetics were almost identical for both complementary hybridization and strong complex formation (Figures 12A-C), showing that hybridization is the rate-limiting step. In contrast, the analysis of binding ATR 1 with appropriate TNF target (Figure 12D) showed that initial binding through hybridization of sense and antisense sequences is relatively rapid and occurs with roughly the same binding constants and rates of formation for both AT and ATR 1 RNAs ($t_{1/2}$ = 10 minutes at 37°C, 10 mM $MgCl_2$ and 50 mM Tris-HCl, pH=8.0). The formation of strong complexes byATR 1 RNA is a slower process ($t_{1/2}$ = 40 min.) than the initial binding. Moreover, radiolabeled ATR 1 RNA can be displaced from the initial complex with TNF1 RNA by the addition of an excess of unlabeled AT RNA, but no such substitution is detected after formation of the strong complex. Rapid strong complex formation in case ATR 16a, ATR 16b and ALR 229 makes these constructs superior over ATR 1.

**F. Delivery of ATR 1 Antisense Constructs to Macrophages for Down-Regulating TNFα**

Effective delivery of biologically active RNA (Malone et al., *Proc. Natl. Acad. Sci. USA* 86:6077-6081 (1989)) and ribozymes (Sioud et al., *J. Mol. Biol.* 223:831-0835 (1992)) has been demonstrated for cells of hematopoietic origin. Lipofectamine (DMRIE/DOPE; Life Technologies, Bethesda, Maryland) has been used to introduce our constructs into murine peritoneal macrophages both *in vivo* and *in vitro* because previous studies had shown it to be superior to a variety of other lipid formulations, with low toxicity (Kisich and Erickson, *J. Leukocyte Biol. Suppl.* 2:70 (abstract) (1991a) and Kisich and Erickson, *FASEB J.* 4:1860 (abstract) (1991b)).

For example, 10 μg of $^{32}P$-labeled hairpin ribozyme (HPR) was complexed with either Lipofectin (DOTMA/DOPE), DMRIE/DOPE, or Lipofectamine and administered i.p. to mice in a volume of 1 ml. Macrophages were harvested after 8 hours and lysed in 97% formamide 5 mM EDTA, 0.1% SDS. The lysates were analyzed by gel electrophoresis and phosphor-imaging to calculate the number of intact HPR molecules per macrophage. The results are presented in Fig. 5.

Macrophages were harvested 3 hr later by peritoneal lavage with HBSS. The exudates were plated at 1 x $10^6$/well in 24-well plates and allowed to adhere for 2 hr, then washed with HBSS to remove nonadherent cells.

After transfection, macrophages isolated as described above, or RAW264.7 cells were stimulated with 100 ng/ml lipopolysaccharide (LPS) to induce TNFα production. Supernatants were sampled at 0, 2, 4, 8, and 24 hr post-LPS stimulation and stored at -70°C until quantitation, which were done by a TNFα-specific ELISA (Biosource International).

**G. Development of Vectors For Endogenous Delivery of ATR 1 Antisense RNAs**

To demonstrate the feasibility of using ATR antisense RNAs in a gene therapy setting, we have incorporated the genes encoding these molecules into eukaryotic expression vectors. The strategy for construction of the mammalian high expression DNA vectors for ATR generation is as follows. Single copy or concatomer copies of the ATR DNA templates are generated by PCR reactions and cloned into the CMV promoter driven pS65T-GFP-C1 vector (Clontech Labs, Palo Alto, CA) downstream from the GFP (green fluorescent protein) expressing region. The T7 promoted vector used for our ATR *in vitro* synthesis was used as DNA template for generation of mammalian cloning sequences. Using a 5' overhang primer, restriction cloning sites for EcoRI and BglII and a eukaryotic stop codon are introduced upstream of the ATR sequence, eliminating the T7 promoter. Single ATR copy expressing vectors are constructed by cloning of the ATR template into the EcoRI-BamHI site in the MCS of the GFP vector. Concatomer copy expressing vectors are generated by creating a head-to-tail multimeric ligation between compatible cohesive ends of BglII head and BamHI tail sites. Head-to-head and tail to tail ligations are inhibited by having BglII and BamHI enzymes present in the ligation mixture (at 100 mM NaCl to avoid "star" activity). Head-to-tail ligations will not be cleaved by these enzymes. The final ladder product is isolated from acrylamide gel electrophoresis and cloned into the BglII-BamHI site of the GFP vector. In-frame directional clones are selected by the characteristic of being cleaved by these two enzymes.

We found that *covalent* circularization (self-ligation) of the hairpin ribozyme domain in ATR 1 antisense RNA is not strictly required for formation of the strong complex with the TNF1 target. Thus, as discussed above, we showed that linear ATR 1 RNA molecules having 5'-[$^{32}$P]-phosphate and 3'-OH ends (i.e, the "R4" form) failed to form the covalent circle R1 RNA. However, this "termini misphosphorylated" RNA molecule also could form strong complexes with the TNF1 target despite the fact that covalent cleavage should obviously result in the strongest padlock. More recent results suggest that a fraction of ligation-competent ribozyme-target complexes are significantly more stable, suggesting that these are covalently linked.

Since the structures of the hairpin ribozyme required for both self-cleavage and self-ligation were shown to be essentially identical (Chowrira et al. (1993), *supra* and Butcher and Burke (1994), *supra*) the covalent bond itself at the cleavage/ligation site presumably does not play a role in stabilization of the catalytically active ribozyme structure. A similar feature has been shown for circularly permuted tRNAs, which can form the same structure even when their nucleotide chains are interrupted by placing nicks in the middle of helices or in anticodon loops (Pan et al., *Science* 254:1361-1364 (1991)). Coaxial stacking across the cleavage sites is probably a factor in stabilizing these structures (Walter et al., *Proc. Natl. Acad. Sci. USA* 91:9218-9222 (1994)). Recent results (Butcher and Burke (1994), *supra*) indicate that the hairpin ribozyme adopts a stable, magnesium-dependent tertiary structure where the sequences adjacent to the cleavage/ligation site are likely involved in non-Watson-Crick base pairing interactions.

EXAMPLE II - **Inhibition of TNFα Secretion by ATR Constructs.**

The ability of various anti-TNFα antisense constructs to inhibit the secretion of TNFα from RAW264.7 cells was determined. Specifically, 2 x $10^5$ RAW264.7 cells were treated with 4.5 μg of the antisense construct ATR 1, ATR 16a, ATR 16b or ALR 229 or control RNA (m101) as described in Section I-F above. The RNA was complexed with Lipofectamine at a 3:3:1 charge ratio for 2 hours in 1 ml DMEM. TNFα levels in supernatents were measured by specific ELISA at

4. Our newer padlock RNA constructs are even better than our original ATR 1; hence the ATR approach can be further improved by design innovations. At the same time, the fact that three or four out of four constructs designed were effective in cell assays indicates that our basic design principles are quite general, rather than being limited to a few instances of accessible target sequences.

EXAMPLE III - Inhibition of TNFα in Mice

We chose our most effective padlock RNA in the cell culture assay to test for *in vivo* efficacy in mice (Fig. 14). As with the *in vitro* assays, ALR 229 was complexed with Lipofectamine and delivered i.p., after which macrophages were recovered and assayed *in vitro* for TNFα production following LPS stimulation as described in the brief description of Fig. 14. Although there was some nonspecific stimulation of TNFα secretion apparently due to the proinflammatory effects of Lipofectamine, mice that had received ALR 229 consistently showed half the level of TNFα production shown by mice that had received a control ATR that was directed at an irrelevant gene (human VCAM-1; the human VCAM-1 ATR is not expected to bind to the mouse VCAM gene or any other gene). We consider this strong evidence that ALR 229 has a significant antisense activity *in vivo*. It also suggests that Lipofectamine, and perhaps cationic lipids in general, may not be the best vehicle for *in vivo* delivery of anti-TNFα agents.

EXAMPLE IV - **Antisense-Mediated Down-Regulation of VCAM.**

In response to injury or infection, leukocytes adhere to endothelial cells lining the walls of blood vessels in the area and proceed to emigrate through the wall and into the affected tissue. This process is mediated by the cytokine-induced expression of several adhesion molecules on the endothelial cell surface, including members of the selectin family (P-selectin, E-selectin) (Lawrence et al., *Cell* 65:859 (1991)) and members of the immunoglobulin family (ICAM-1, ICAM-2 and VCAM-1) (Oppenheimer-Marks et al., *J. Immunol.* 147:2913 (1991)). VCAM-1 is induced by IL-1, IL-4 and TNF, and reaches maximal levels 10-14 h after cytokine treatment, remaining elevated for up to 72 h (Rice and Bevilacqua, *Science* 246:1303 (1989) and Masinovsky et al., *J. Immunol.* 145:2886 (1990)).

3792 as well as the complementary sequence of VCAM mRNA (with start codon underlined) are as follows:

ISIS 3792 3'-GCTGTCGTTGAATTTTACGG-5'

VALR1 antisense sequence 3'-GCUGUCGUUGAAUUUUACGG-5'

VCAM-1 mRNA target sequence

5'-CGACAGCAACUUAAAAUGCCUGGGAAGA-3'

To the antisense sequence ribozyme and linker sequences were appended so that the expected structure of the resulting construct upon binding to its target would be as shown in Fig. 7. The VALR1-target complex contains approximately one full turn more of sense-antisense duplex than triplex. In this regard, if there is no excess duplex over triplex, there can be no linkage, as the turns of the third strand would unwind the turns of the duplex around the target.

Synthesis of the complete VALR1 ATR RNA, designated VALR1, was accomplished by T7 transcription of appropriate DNA templates as described above for the TNFα system. For a target site, we used VALRT 1, a 642-nt partial transcript of VCAM mRNA fused with a piece of pSP-luc+NF vector sequence. This RNA contained a 20-nt sequence around the AUG codon complementary to the antisense domain of the VALR 1 molecule (see figures), located 247 nucleotides (nt) downstream of the target 5'-end and 375 nt upstream of the 3'-end.

VALR 1 was even more active than ATR 1 (TATR 1) in self-processing (self-cleavage) of its precursor RNAs but, in contrast to ATR 1, self-processed linear VALR 1 RNA showed very little ability to circularize in the absence of a target. However, self-processing of the pre-VALR 1 RNAs in the presence VALRT 1 target resulted in formation of circular VALR 1 species, as detected by denaturing gel-electrophoresis. We also showed that VALR1 RNAs can form a strong complex with VALRT 1 RNA, stable after gel-electrophoresis in denaturing conditions (8 M urea, 2 mM EDTA, 45°C for 2 hr.).

For testing the ability of VALR1 to inhibit VCAM-1 expression, control RNAs are synthesized that lack the catalytic hairpin ribozyme moiety or contain an inactive, mutated version of it, as well as ALRs that lack the target binding site.

overexpression is associated with tumor progression or metastasis, and whose down-regulation is expected to normalize growth control. Although many such proteins will find use in the present method, we will herein use the HER-2 gene as the antisense target. Repression of HER-2 in mouse tumors leads to suppression of tumor growth and longer survival of the mice; hence it is an attractive target for antisense therapy. The structure and sequence of the binary agent will be optimized through biochemical assays for tightness and specificity of binding, and if necessary through selection from randomized sequences. Then its effectiveness at blocking translation will be tested, first in an *in vitro* translation system and then in cultured tumor cell lines.

Clinical trials of antisense therapy for cancer are underway for at least two target genes relevant to cancer. However, a major limitation of this approach, and indeed of all drug therapy in current use for cancer, is the lack of ability to target the drug specifically to cancer cells. Thus, the goal of the presently described work is to employ a novel method for providing antisense therapy of breast cancer with this needed element of cell specificity. Treatment of breast cancer patients based on this approach will provide increased effectiveness and greatly decreased side effects. As described above, we have designed a ribozyme-based antisense agent intended to covalently link itself around its target mRNA after hybridizing to it. This linkage provided much greater strength of binding and potency in blocking translation. Here, however, we extend that approach to make the linkage dependent on the presence of c-myc, a nuclear protein present in elevated levels in breast cancer cells which binds to a specific DNA sequence. Thus, the antisense oligonucleotide binds to the mRNA target, wrapping around it due to the helical winding of the sense-antisense duplex. By bringing the ends closer together and reducing the conformational entropy of the molecule, this binding to the target permits the ends to pair with each other in an additional short, weak duplex which contains a binding site for c-myc (with its cofactor max) (see e.g., Fig. 1C, D and H and Fig. 8 for a schematic illustration thereof). The binding energy of this sense-antisense complex by itself is too weak to suppress the target gene. However, if c-myc is present at elevated levels, it binds to the weak duplex, stabilizing it and "locking" the complex together by virtue of their helical interwinding. The antisense oligonucleotide can

minimal toxicity at therapeutic levels. We present here a novel approach to achieving the needed specificity by having two levels of recognition, analogous to a binary weapon. Thus, instead of attacking all cells that, say, are actively dividing, or that possess a particular cell-surface marker, the proposed agents down-regulate an appropriate target gene only upon binding to some molecule that is present mainly or exclusively in the cancer cell. The great power of the method is that the triggering molecule and the target gene can be (but are not required to be) completely unrelated. The target gene can potentially be any gene, even a housekeeping gene, although the highest level of specificity is achieved by choosing a target gene that is active mainly or exclusively in cancer cells. Here, we choose as ~~the triggering molecule the phosphoprotein product of the c-myc gene, and HER-2~~ as the target gene. The mechanism of triggering is based on the padlock idea but where topological linkage requires stabilization by binding of a separate agent, the "clasp." We propose to use c-myc as the clasp.

A novel feature of this two-tiered approach is that its effectiveness should be proportional to the product of the concentrations of two elements that are more abundant in breast cancer cells: c-myc protein and HER-2 mRNA (or DNA, in the antigene version of the approach). Since the HER-2 gene is often amplified by as much as 10-fold, and the abundance of c-myc may be similarly elevated in some cancer cells, the therapeutic index of our approach is expected to be much larger than current therapies.

This approach lends itself well as an adjunct to other, standard therapies. Because c-myc overexpression is associated with poor prognosis of breast cancer, it might be expected that the resistance of tumor cells to standard chemotherapeutic agents may correlate with c-myc expression. This was found to be the case with cis-platin: Treatment of cis-platin-resistant cells in culture with c-myc antisense oligonucleotides reversed the resistance, possibly by increasing uptake of cis-platin; and even in nonresistant cells that express c-myc, there was a synergistic cytotoxic effect (Mizutani et al., *Cancer* 74:2546-2554 (1994)). Synergistic effects have also been seen for combination c-myc-P53 antigene therapy (Janicek et al., *Gynecol. Oncol.* 59:87-92 (1995)).

(Noonberg et al., *Nucleic Acids Res.* 22:2830-2836 (1994)) and transcription in vitro (Ebbinghaus et al., *J. Clin. Invest.* 92:2433-2539 (1993)).

Full-length c-myc binds sequence-specifically to its target site only as a heterodimer with the related protein max (Blackwood and Eisenman (1991), *supra*). To avoid having to use both c-myc and max in our cell-free assays, we will take advantage of the fact that a truncated version of c-myc consisting of the basic, helix-loop-helix, and leucine zipper domains binds to the same site as the c-myc/max dimer.

Candidate antisense constructs will be tested for c-myc-dependent binding and translation inhibition first in a cell-free system and then tested for biological effects on cultured breast cancer cells. The goal of these prototype experiments will be a general procedure for down-regulating any gene in the presence of any given protein.

To accomplish the above, the following will be performed. First, the sequences HER-5', HERMYC1, and HERMYC2 as shown in Fig. 8 will be synthesized in both phosphodiester and phosphorothioate forms. If finding the right conditions for making the padlock highly sensitive to the presence of the c-myc clasp turns out to be difficult, we will synthesize an analogous oligonucleotide containing the E. coli lac operator sequence in place of the c-myc-max binding site. This will permit optimization to be done with a single, easily available protein trigger, the lac repressor. If necessary, the lengths of linker and helical regions will be optimized by *in vitro* selection. The optimal lengths of helical segments determined for the lac repressor are likely to be also optimal for c-myc used as the clasp.

Next, the binding characteristics and kinetics by polyacrylamide gel-shift assay using 5'-$^{32}$P-labeled padlock oligonucleotides will be verified. The DNAs will be annealed by heating and slowly cooling to 2°C, then electrophoresed at different temperatures ranging from 4°C to 37°C in the presence of $MgCl_2$ to mimic intracellular concentrations of available $Mg^{2+}$.

Next, the stability of the complexes will be verified by measuring the melting temperatures of padlock oligonucleotide HERMYC1 with target HER-5' and HERMYC1 with HER-5' by UV absorbance, ramping the temperature from 2° to 45° and back down at 0.5°/min in 25 mM Tris HCl (pH 7.4), 100 mM KCl, 1 mM

necessary in the presence of 0.1% SDS). DNAs that bind in the absence of c-myc (thus bypassing the clasp) will be eliminated by passing this selected pool through the column once again in the absence of c-myc and collecting the flow-through. This depleted pool will be amplified by PCR and then subjected to further rounds of selection. The concentration of c-myc will be reduced gradually to increase the selection pressure for ligands for which c-myc has a strong "clasping effect." The pool will be sequenced *en masse* to see whether the search is narrowing, and when specific sequence patterns emerge the pool will be cloned, individual colonies sequenced, and consensus sequences derived. These will be individually synthesized and tested for their ability to function as switches.

*In vitro* translation assays for ribozyme obstruction will be performed as described in the above example, using a fusion construct between HER-2 mRNA and luciferase. Controls will include oligos having scrambled and sense sequences in place of the antisense sequence, and clasp sequences that cannot bind c-myc. Before addition of an rabbit reticulocyte lysate translation mixture, oligos will be incubated in 50 mM Tris acetate (pH 7.5)/10 mM magnesium acetate, either alone or in the presence of a 10- to 40-fold molar excess of *in vitro*-transcribed (T7) HER-2-luciferase fusion constructs. Luciferase will be assayed according to the instructions from the translation kit manufacturer (Promega). A T7 promoter-luciferase expression plasmid lacking the target sequence will be used as a further control.

For cell testing, we will use the phosphorothioate versions of the successful oligonucleotides from the previous experiments. We will then expose estrogen-dependent MCF-7 breast cancer cells or other appropriate c-myc-overexpressing cells to this oligonucleotide or control oligonucleotides with and without estrogen, and assay for HER-2 mRNA, protein production, and cell proliferation. If appropriate, we will compare with estrogen-independent MDA-MB-231 cells, which constitutively express high levels of c-myc. We will use a cationic lipid vehicle: lipofectamine or lipofectin (Life Technologies), or dioleoylphosphatidylethanolamine (DOPE) together with a cationic cholesterol derivative (DC cholesterol) previously shown to be less toxic for down-regulation of HER-2 expression through a gene therapy approach (Hung et al. (1995), *supra*). We will also try the unmodified

ability of RNA to catalyze its own cleavage and rejoining reactions. By taking advantage of RNA catalysis, we can eliminate requirements for all protein factors except an RNA polymerase and reduce the number of tube openings. Additional innovations in hybrid capture and wash procedures further increase the speed and ease of automation. The procedure lends itself to closed-tube, multiplex fluorescent detection during amplification, permitting rapid screening of many different targets while minimizing risks of exposure to pathogens or laboratory contamination by amplified targets.

The ultimate goal is to design a scheme for nucleic acid-based diagnostics that can be used to detect either DNA or RNA, is sensitive, rapid, requires no thermal cycling, and readily lends itself to automation. Our proposed scheme is based on the use of RNA catalysis and Qβ-replicase for amplification. This RNA-dependent RNA polymerase will catalyze exponential replication of an RNA molecule possessing appropriate end sequences, without the need for primers or thermal cycling. Following the work of Tyagi et al., *Proc. Natl. Acad. Sci. USA* 93:5395-5400 (1996), we separate the end sequences by dividing the substrate RNA into two halves, called replication probes; hence amplification can proceed only if the two replication probes are ligated together (Fig. 25). They are designed to hybridize to adjacent sites on the target RNA, so that ligation (and therefore amplification) are dependent on the presence of the target. Background is minimized by capturing the target on a solid substrate through hybridization with capture probes, followed by washing away non-hybridizing RNAs and then release from the substrate. The purified target is hybridized to the replication probes, which are then ligated and amplified. In one embodiment, this procedure requires three enzymes: ribonuclease H (RNase H) to release from the solid support, DNA ligase (which acts on double helical RNA) to ligate the replication probes, and Qβ-replicase. In our proposal we utilize target-dependent RNA catalysis to substitute for both the nuclease and ligase proteins (Fig. 26). By incorporating their catalytic functions into the RNA probe molecules, we eliminate the need for any protein enzymes other than the replicase, and increase the specificity of target recognition by using shorter hybridization probes. We use the hairpin ribozyme for this purpose because it efficiently catalyzes both cleavage and ligation.

which is not sufficient for many desired applications. Moreover, the most sensitive hybridization assays usually lack features required for routine applications —safety, economy, convenience and speed.

One solution of this limited sensitivity is exponential amplification of the target sequence. This can be carried out by either temperature-cycle assays such as the polymerase chain reaction (PCR) and ligation chain reaction, or isothermal procedures such as transcription-mediated amplifications and the restriction nuclease/DNA polymerase method. Alternatively, hybridization can alter another component of the reaction so as to make it amplifiable; examples include linear amplification methods such as induction of an enzyme reaction to produce a fluorescent product, or exponential amplification of reporter RNA through the use of Qβ-replicase (Chu et al., *Nucl. Acids Res.* 14:5591-5603 (1986)).

In practice, PCR, although providing very high sensitivity, has several limitations: (1) the occurrence of false positives generated by hybridization of primers to homologous sites in non-target DNA, (2) the presence of PCR inhibitors in specimens, and (3) its inability to directly amplify RNA due to its thermal lability. These drawbacks, together with market considerations of license fees for the use of patented PCR technology and the significant cost of thermal cyclers, have stimulated a search for alternatives. Prominent among the newer techniques is the isothermal, exponential amplification of recombinant RNA probes by Qβ-replicase. Although the PCR and the Qβ-replicase assays have similar sensitivity (Lizardi et al., *Biotechnology* 6:1197-1202 (1988)), the Qβ-replicase assay is simpler, faster and less expensive than PCR. The substrate for Qβ-replicase is RNA rather than DNA, and RNA combines the dual functions of hybridization probe and amplifiable reporter. In this proposal we make use of a unique capability of RNA, autocatalysis, to eliminate the need for two protein enzymes used in a promising current amplification scheme (Tyagi et al. (1996), *supra*) employing Qβ-replicase.

Qβ-replicase is an RNA-dependent RNA polymerase from the coliphage Qß. It is capable of replicating the single-stranded Qß RNA genome in infected cells while ignoring the huge excess of bacterial RNA, and similar specificity has been observed in in vitro assays (Haruna and Spiegelman, *Science* 150:884-886 (1965)). As little as one molecule of template RNA can in principle initiate its exponential

amplified unless they hybridize to their target (EP-A-707 076 and Tyagi et al. (1996), *supra*), so-called "smart" probes. These permit very low background in hybridization assays, since signal generation is strictly dependent on the presence of target RNA.

One smart probe approach is to divide the amplifiable reporter RNA into two separate molecules neither of which can be amplified by itself, because neither contains all the elements of sequence and structure that are required for replication. The division site is located in the middle of the embedded probe sequence. When these "binary probes" are hybridized to adjacent positions on their target, they can be joined to each other by incubation with an appropriate ligase, generating an amplifiable reporter RNA. Nonhybridized RNA probes on the other hand, because they are not aligned on a target, have a very low probability of being ligated.

In another approach (EP-A-707 076), latent Qβ-replicable template has been created by extending on the 5' end of MDV-1 RNA resulting in inhibition of its replication by Qβ-replicase. A ternary hybrid formed between this latent substrate, a second RNA probe, and a target RNA produces an autocatalytic RNA structure (hammerhead ribozyme) which cleaves the 5' extension from the latent template in the presence of divalent cations, thereby converting it to an efficiently replicating form and effecting its release from the support. This approach is interesting as a first attempt to use the catalytic potential of RNA molecules in nucleic acid-based diagnostics. However, it has two disadvantages: It is limited to target sequences that contain the element GAAA (required to generate an active hammerhead ribozyme) and unintended spontaneous RNA cleavage (and, therefore, activation of replication) can readily occur through transesterification, catalyzed by the same divalent metal ions that are required as catalytic cofactors for the ribozyme.

In this approach, we combine the best features of these two approaches by employing a hairpin ribozyme construct for the target-dependent-ligation of two halves of a split substrate for Qβ-replication. Our approach imposes no sequence restrictions on the target RNA, and because spontaneous ligation can be made much rarer than spontaneous RNA cleavage, the background of false positives should be very low. Moreover, because the hairpin ribozyme can catalyze both ligation and cleavage (see below), it can play dual roles of target-dependent ligase and specific

ligation of nonadjacent substrates is utilized in our scheme as diagramed on Fig. 25 and described below.

**Catalysis by freezing and ethanol.** Extensive characterization of individual forms generated by self-processing of HPR T7 transcripts revealed that either freezing alone or exposure to >40% ethanol alone results in ligation of linear forms in the absence of divalent cations (Kazakov et al., submitted)). Actual freezing was required, since ligation did not occur in supercooled but unfrozen solutions at -21°C.

Both freezing and ethanol treatment were able to produce yields of ligated product (up to 85%) that exceed the yield (~50%) in normal aqueous solution. Since the key step in our scheme for amplification by Qβ replicase is ligation of the replication probes (see below), we have the option of incorporating a freezing step if needed for maximal sensitivity.

We describe here procedures for analysis of sample of cells to be analyzed for pathogens through detection of mRNA; with an initial denaturation step, a similar protocol would work for DNA targets, suitable; for example, for detection of genetic variants. The general scheme is outlined in Fig. 26. The first step involves capture of the target on a solid substrate using a probe that is complementary to the desired target, so-called hybrid capture. The procedure for hybrid capture is analogous to that used by Tyagi et al. (1996), *supra*, except for the composition of buffer solutions and the structure of the capture probe. The capture probe consists of a sequence complementary to the target RNA, a short linker, the substrate sequence for the HPR, another linker, and a terminal biotinylated nucleotide (Fig. 26). It needs to be composed of RNA residues in the region of the cleavage site; the rest can be DNA or a nuclease-resistant analog. In our first version, it will be all RNA for simplicity.

A sample of cells to be analyzed for pathogens is dissolved by incubation in 5 M guanidine thiocyanate for 60 min at 37°C. This treatment lyses cells, inactivates enzymes, frees DNA and RNA from intracellular structures, and weakens RNA secondary structures (Pelligrino et al., *Biotechniques* 5:452-460 (1987)). Lysates are adjusted to reduced guanidine thiocyanate concentration (Buffer A: 2M guanidine thiocyanate, 400 mM Tris-HCl (pH 7.5), 0.5% sodium N-

HCl (pH 8), 100 μM ATP, 600 μM CTP, 600 μM UTP, and 600 μM GTP) and Qβ-replicase (6 μg, Vysis).

From this point forward, no further manipulations are required; the remaining steps proceed in sequence automatically. RNAs 4 and 5 hybridize to a pair of sites located 50 nt from the binding site of the HPR domain E, and the 2'-3'-cyclic phosphate end of RNA4 and the 5'-OH end of RNA5 make up a substrate pair that can bind and be ligated by the HPR. They can form a complex through looping of the target RNA as shown in Fig. 26. This complex mimics the structure of the native HPR in its cleaved form, and leads to ligation with an efficiency of approximately 50%.

Synthesis of RNAs with appropriate ends. The replication probes must have 5'-OH and 2',3'-cyclic phosphate ends in order to be ligated by HPR domain E. The simplest way to achieve this is the automatic scheme shown in Fig. 28. Two RNAs, each containing the full HPR substrate domain but only one Qβ-recognition sequence, are provided to the target-hybridized domain E. Whenever one of these RNAs hybridizes to the target and occupies the substrate binding site it will be cleaved (Fig. 28). Due to the high off-rate for the cleaved products, these products will dissociate from domain E, permitting another uncleaved RNA to bind and be cleaved. After some time, enough RNAs will be cleaved that with reasonable probability a left-hand and a right-hand replication probe will bind to the same Domain E and since they will now have the appropriate ends, they will be ligated. Qβ will then rapidly amplify these molecules. The process is aided by the fact that the large cleavage products will remain near Domain E due to hybridization to the target, whereas the small fragments will diffuse away.

The above described scheme will be tested using as target a sequence from the pol gene of HIV-1. Domain E will bind to nt 4668-4682 of the HIV genome (Tyagi et al. (1996), *supra*), the capture probe will bind to nt 4716-4760, the left replication probe to nt 4577-4588, and the right to nt 4607-4618. We will first test for the efficiency of the individual steps by using $^{32}$P-labeled target or probes and following the recovery at each step.

We must also design a derivative of HPR that has catalytic activity dependent on target binding. In the normal HPR, the catalytic domain (E) is stabilized by a short

Having obtained good candidates for the target-dependent catalytic moiety, the next step will be to demonstrate its ability to cleave a separate RNA (in trans) if the two RNAs are hybridized to adjacent sequences on a target RNA. For this purpose, we will construct DNA templates for the transcription of target RNA spanning the region of HIV to which all the probes bind (nt 4500-4800). Using the biotinylated probe shown in Fig. 27, we will demonstrate capture of this (labeled) RNA on streptavidin-paramagnetic beads, following the procedures given above and in Tyagi et al. (1996), *supra*). Upon adding RNAs 2 and 3, we will test for release of the labeled RNA from the beads. If necessary to achieve release of a reasonable fraction of bound complexes (50% would be ample), we will adjust reaction conditions (temperature, magnesium ion concentration, etc.) and the length of the oligo(U) spacer on the capture probe (Fig. 27).

The next step is to test the ability of the target-tethered ribozyme to ligate the Qβ replication probes (constructed with appropriate ends as described above) and achieve amplification in the presence of the replicase. If necessary, we will test the ability of Qβ replicase to use our probes as substrates by synthesizing a short complementary RNA to hold the ends together and performing ligation with DNA ligase.

Finally, we will combine the above steps and test the ability of the ribozyme to switch from cleavage of the capture probe to ligation of the replicase probes in the presence of the target. If needed, we will adjust lengths and/or AT/GC composition of helical stems surrounding cleavage/ligation sites if needed.

To test for sensitivity and background, prepare simulated diagnostic samples by using a dilution series of T7-transcribed target RNA so that samples will contain as little as 1 molecule of target. α-[$^{32}$P]-CTP will be included in the Step 3 (see Fig. 25) and aliquots will be removed at 1-min intervals beginning 10 minutes into the reaction, until the reaction has proceeded for 35 min. Each aliquot will be precipitated by acid (addition of 400 μl of 360 mM phosphoric acid, 20 mM sodium pyrophosphate and 2 mM EDTA (Tyagi et al. (1996), *supra*). The precipitates will be collected a nylon membrane (Zeta-Probe, BioRad) through a vacuum manifold, washed, and quantitated by autoradiography or using a Phosphorimager (Storm 840, Molecular Dynamics).

solid fibers, capillaries permit reaction and washing steps to be done without pipetting, much as DNA is synthesized on a solid-phase column. Detection could still be performed through fiberoptic means, since the capillary would provide for total internal reflection of exciting and emitted light at least as efficiently as with solid fibers.

**Enhancement of ligation by freezing.** The efficiency of ligation can be increased from about 50% to about 85% by freezing the solution to -5° for 15 min. We will try this procedure to see if the added efficiency warrants the additional step.

**Relaxing stringency.** The procedures described above provide the maximum level protection from false positive signals, by requiring independent target recognition by ~~five separate RNA molecules in order for amplification to proceed. Such high~~ "stringency" may not be necessary, especially for certain applications. Thus, it may not be necessary to have the replication probes bind to the target, as long as the catalytic activity of Domain is strictly dependent on target binding. The replication probes could be tethered to Domain E either covalently or via hybridization or metal coordination to provide efficient target-dependent ligation in this case.

**Sensitivity.** The sensitivity of this assay depends on the efficiency of all the steps leading to amplification of the target RNA. In the version of Tyagi et al. (1995), *supra*, the proportion of target molecules that resulted in amplifiable product was 2.5%. The main source of loss was the ligation step using T4 DNA ligase, which operates inefficiently on RNA and produced only 8% ligated product. In contrast, typical HPR derivatives such as HPR1 exhibit ligation efficiencies of 50% and as high as 85% upon freezing. Even with an overall efficiency of 2.5%, Tyagi et al. (1995), *supra*, could detect the presence of 100 but not 10 molecules of target. We anticipate that the higher yield of HPR ligation will increase the sensitivity to less than 10 molecules.

The other important characteristic of an effective diagnostic technique is the background of false positive events. False positives in this procedure come from ligation events that occur in the absence of hybridization to the target. Qβ-replicase can occasionally continue polymerization across a gap between replication probes that somehow are juxtaposed to each other, resulting in an amplifiable reporter RNA. Tyagi et al., (1995), *supra*, found that the only significant source of such

to the binding sites of the replication probes, and also dependent on target binding for ligation activity.

The above discussion illustrates one manner in which binding of a target can activate a latent ribozyme to produce a signal for detecting the target molecule. Another embodiment of this idea is shown in Figure 32. Here binding of a nucleic acid target molecule stabilizes the structure of a hammerhead ribozyme, leading to cleavage of its substrate strand. Such cleavage can elicit a signal, as for example in the case of the left-hand construct, if the substrate strand is tethered to a solid support at one end and has a signal group at the other end, such as a fluorophore or biotin. Binding of a target molecule leads to separation of the signal group from the solid support, where it could be quantitated by standard methods upon removal from the solution of the solid support. Alternatively, one end may be attached to a fluorescent group and the other end to a quencher of fluorescence such that cleavage causes dequenching and fluorescence appears (Walter and Burke., RNA 3:392-404 (1997)). Because the target nucleic acid molecule does not have to be cleavable by the ribozyme, there are no limitations on its sequence.

A more general embodiment of this idea is shown in Figure 31. Here the target molecule can be potentially any molecule of interest, including proteins, small molecules, and metal ions. The binding site for the target molecule comprises the ends of two strands of the hammerhead ribozyme as shown; the sequences of those ends are selected from combinatorial libraries of DNA or RNA sequences to bind specifically and tightly to the target of interest (Tang and Breaker, RNA 3:914-925, 1997). Binding of the target molecule stabilizes the active conformation of the ribozyme and produces cleavage of the substrate strand.

EXAMPLE VII - Methods for Labeling Antisense and Triplex Forming Oligonucleotides with Platinum.

All stock solutions were prepared in water deionized by a Milli-Q apparatus (Millipore), and then filtered through 0.22 $\mu$m filter units (Nalgene). The contents of common stock buffer and solutions used were as follows:

20% AUB: 19% acrylamide / 1% N,N'-methylene-bis-acrylamide / 8.8 M urea / 1 x TBE.

2 $\mu$l $H_2O$

1 $\mu$l 10xPNK buffer (Promega)

5 $\mu$l [$\gamma$-32P]ATP (10 $\mu$Ci/$\mu$l) (Amersham)

1 $\mu$l T4 Polynucleotide kinase (10 U/$\mu$l) (Promega)

Mix and incubate at 37°C for 30 min.

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$^{32}$P-labeled oligonucleotide species were purified (and analyzed) by electrophoresis through 20% denaturing polyacrylamide gels. Immediately before loading onto the gels, the solutions were mixed with equal volumes of 2xFLS, and heated for 2 min at 95°C. Individual oligonucleotide bands were located by autoradiography and isolated from the gels as described above.

**Diethylenetriamine catalyzes platination of oligonucleotides.** Various reaction mixtures (total volume 10 $\mu$l) were combined as following:

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1 $\mu$l of $^{32}$P-labeled oligonucleotide

14 $\mu$l of 150 $\mu$M of the same non-radioactive oligonucleotide

2 $\mu$l of 5-50xTAE buffer

0.3 $\mu$l of 0.01-1 mM $K_2PtCl_4$

0.3 $\mu$l of 0.1-15 mM dien in 10-100xTAE

0.3 $\mu$l of 0.5-500 mM NaCl, or 0.05-50 mM KI, or 0.075-7.5 mM DMSO, or 0.05-5 mM thiourea

0.7 $\mu$l of $H_2O$

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The reaction conditions, including reagent concentrations, temperatures and times of incubation, are indicated in the figure legends. The platination reactions were stopped by the addition of 1$\mu$l of 1 M NaCl (Brabec et al., 1994), mixed with an equal volume of 2xFLS and analyzed by electrophoresis on 20 % denaturing polyacrylamide gel.

In agreement with literature data (Wherland et al., 1973; Kasianenko et al., 1995) regarding low reactivity of anionic $[PtCl_4]^{2-}$ towards nucleic acids, we found that $K_2[PtCl_4]$ at 30 $\mu$M concentration does not affect the electrophoretic mobility of the 10 $\mu$M oligonucleotide TT or its derivative TST having single phosphorothioate

between $[(dien)PtCl]^+$ and the POS sulfur is known to be nearly independent of the $Cl^-$ concentration whereas the reaction with guanine N7 can be completely inhibited at high concentrations of NaCl (Reedijk, 1991; Slavin et al. 1994). Indeed, we observed inhibition of the platination of both TT and TST oligonucleotides at 100 mM concentration NaCl (Fig. 21, lanes 11 and 22). Moreover, a pre-incubation of the [chloroplatinate-dien] mixtures at different conditions before mixing with the oligonucleotides did not accelerate the platination of oligonucleotides (data not shown). These results suggest the second pathway.

Interestingly, the presence of 1 mM NaCl in the reaction mixture does not reduce the platination yield (Fig. 21, lanes 9 and 20) but, in the case of TT oligonucleotide, makes the product band sharper than without NaCl (Fig. 21, lanes 9 and 3). Since the mobilities of the products formed by platination of both TT and TST oligonucleotides were identical even under the high resolution 20% gel-electrophoresis, we assume that the structure of the tethered platinum groups is the same in both cases. Platination reactions carried out in the presence of ligands forming very strong bonds with platinum, such as anionic $I^-$ (Fig. 21, lanes 4-7 and 15-18), and neutral thiourea, did not change the gel-mobility of the product. Therefore, these ligands cannot compete with dien (a very strong chelating agent) for the binding of the tethered platinum groups. However, they can inhibit the initial reaction of platinum binding to the oligonucleotides at high concentrations (Fig. 21, lanes 7 and 18). We suggest that the platinum group attached is $[(dien)Pt]^{2+}$, which is known to form chemically inert and thermodynamically stable adducts through both sulfur of POS or N7-position of guanine.

**What metal binding center is more reactive: POS or $G_n$ clusters?** The longer reaction time at 45° C provides a higher yield of platination of both TT and TST oligonucleotides after 2 h incubation (Fig. 22, lanes 4 and 10), than that after 1h (used for the experiments presented on Fig. 21), but showed no further increase after 4 h incubation (Fig. 22, lanes 6 and 12). It also revealed that the platination of TST (Fig. 22, lanes 10 and 12) proceeded more specifically than of TT (Fig. 22, lanes 4 and 6), which showed formation of additional product bands. Interestingly, platination of a similar model system, S-guanosyl-L-homocysteine (GSH), showed a kinetic preference for the cysteine sulfur modification over the guanine N7

3 mM dien

10 mM Tris-OAc, pH 7.5

1 mM $Na_3EDTA$

1 mM NaCl

Incubation for 2h at 45° C.

The contents of the optimal platination mixtures (10 μl) is:

1 μl of $^{32}P$-labeled oligonucleotide in 1xTE

1 μl of 100 μM non-radioactive oligonucleotide in 1xTE

2 μl of 150 μM $K_2PtCl_4$*

2 μl of 15 mM dien in 50xTAE

2 μl of 5 mM NaCl

2 μl of $H_2O$

(* fresh solution in $H_2O$ pre-incubated for 1 h at 45° C before adding to the reaction mixture).

In looking for the optimal conditions, we found prominent effects of a variety of components of reaction mixtures on the platination yield and number of products formed.

**Dien.** The higher the dien concentration, the better the yield of the platination. Compare, for example, the product yields in the presence of 1 mM dien and 3 mM dien (Fig. 23, lanes 2-5). However, at concentrations above 3 mM the reaction yield declines presumably because of the precipitation of oligonucleotides.

**Pt and oligonucleotide concentrations.** For preparative bimolecular (second-order) reactions the concentrations of both components are important. In our case the amount of radioactive platinum (and therefore its concentration) is a limiting factor. We can not resolve this limitation by using an excess of oligonucleotide components (making of pseudo-first order reaction) because of possible oligonucleotide precipitation and problems with isolation of platinated oligonucleotides. We determined the allowable concentration range for $K_2PtCl_4$ to be 3 to 100 μM in 10 μl reaction mixtures (0.03 to 1 nmoles platinum); below that level we did not detect any platination of oligonucleotides for reasonable reaction time, whereas above that level we observed fast non-specific modification and precipitation of

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WHAT IS CLAIMED IS:

1. A nucleic acid molecule or analog thereof which specifically binds to a target nucleic acid through a sequence which is substantially complementary to a sequence of the target nucleic acid, said nucleic acid molecule or analog thereof being capable of topologically linking to at least a portion of said target nucleic acid through the interaction between the 5' and 3' ends of said nucleic acid molecule or analog thereof, said topological linkage effectively reducing the efficiency of transcription or translation of said target nucleic acid.

2. The nucleic acid molecule or analog thereof of Claim 1 which comprises catalytic RNA.

3. The nucleic acid molecule or analog thereof of Claim 2, wherein said catalytic RNA is a catalytic region derived from the hairpin ribozyme.

4. The nucleic acid molecule or analog thereof of Claim 2, wherein said catalytic RNA is connected to said sequence which is substantially complementary to a sequence of the target nucleic acid through a bond or a linker.

5. The nucleic acid molecule or analog thereof of Claim 2 which further comprises a triplex-forming region that interacts with the duplex formed between said nucleic acid molecule or analog thereof and said target molecule through the formation of Hoogsteen or reverse Hoogsteen bonds.

6. The nucleic acid molecule or analog thereof of Claim 5, wherein the triplex-forming region forms one fewer helical turn than said duplex so as to ensure topological linkage of said nucleic acid molecule or analog thereof to said target nucleic acid.

7. The nucleic acid molecule or analog thereof of Claim 1, wherein said interaction between the 5' and 3' ends of said nucleic acid molecule or analog thereof is by covalent bonding, Watson-Crick pairing, Hoogsteen bonding, reverse Hoogsteen bonding or other noncovalent interaction.

16. The nucleic acid molecule or analog thereof of Claim 9 which comprises at least one phosphorothioate internucleotide linkage.

17. The nucleic acid molecule or analog thereof of Claim 9 which comprises a competing internal structure which is competed out by binding of said locking molecule.

18. A method for inhibiting the transcription or translation of a target nucleic acid molecule, said method comprising:

contacting said target nucleic acid molecule with a nucleic acid molecule or analog thereof according to Claim 1 or Claim 9, wherein said nucleic acid molecule or analog thereof becomes topologically linked to said target nucleic acid molecule, thereby inhibiting transcription or translation therefrom.

19. A method of covalently bonding a platinum molecule to an oligonucleotide, said method comprising:

combining said oligonucleotide with (1) a platinum donor molecule and (2) a positively charged polyamine molecule under conditions that allow covalent bonding of said platinum molecule to said oligonucleotide,

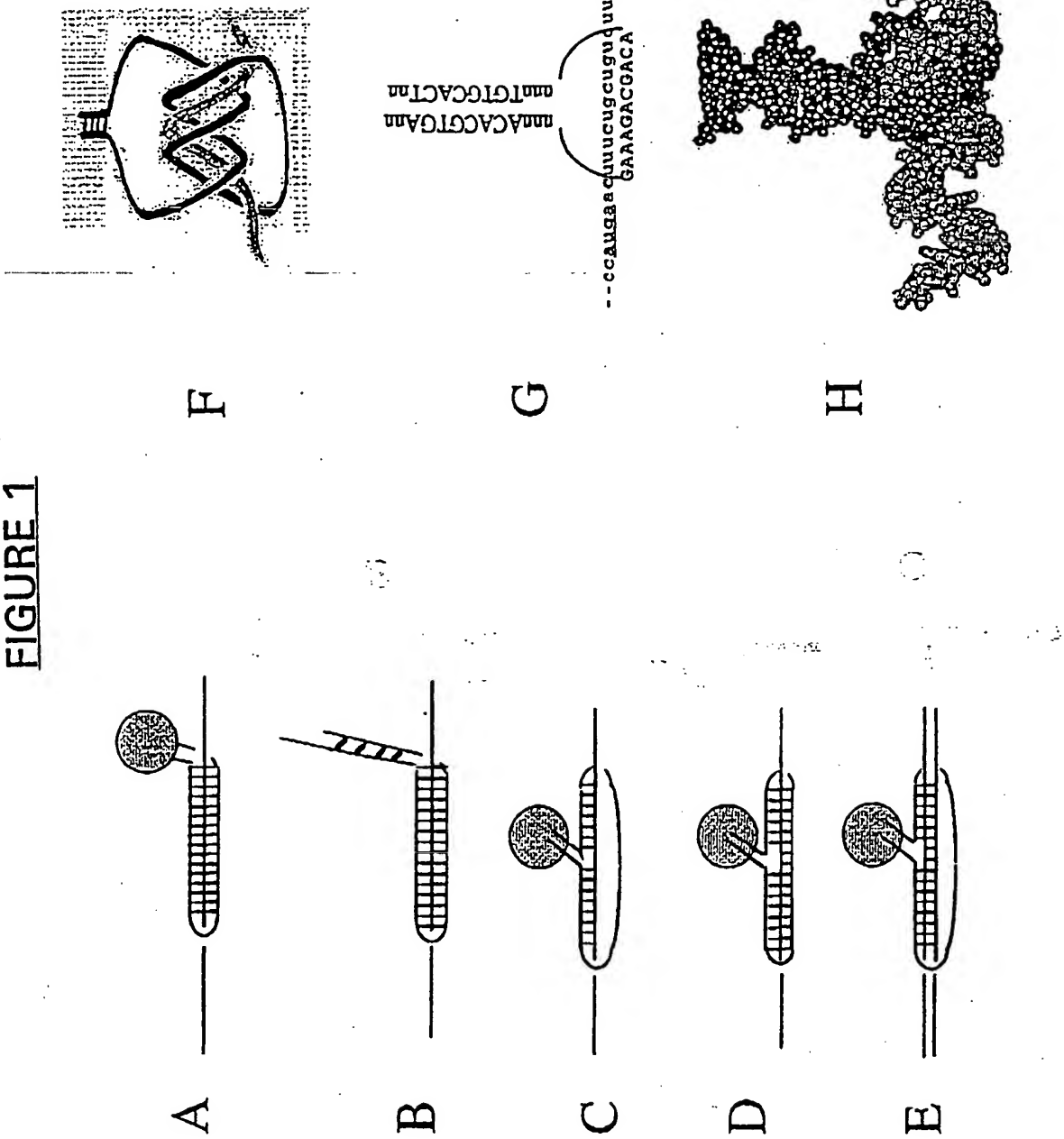
wherein said platinum molecule becomes covalently bound to said oligonucleotide.

20. The method according to Claim 19, wherein said platinum donor molecule and said positively charged polyamine molecule are complexed proir to combining with said oligonucleotide.

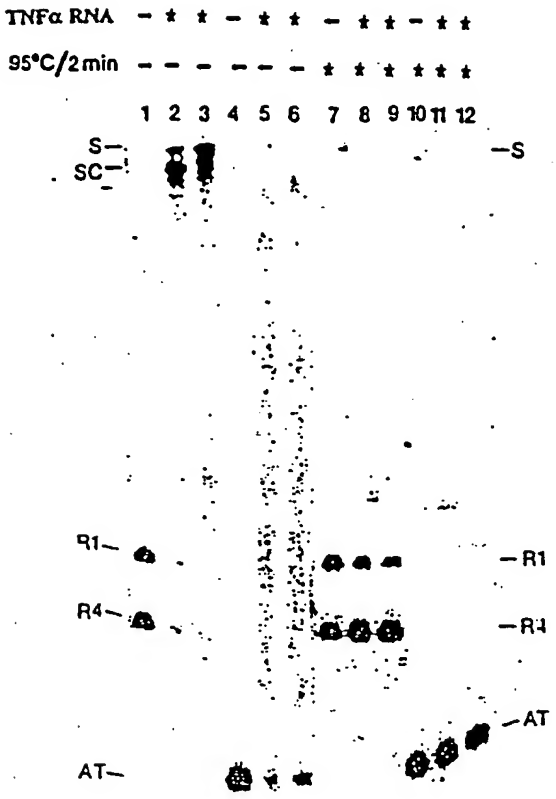
21. The method according to Claim 19, wherein said platinum molecule becomes covalently bound to a sulfur molecule or an N7 of a guanine residue of said oligonucleotide.

22. An oligonucleotide obtained by the method according to Claim 19.

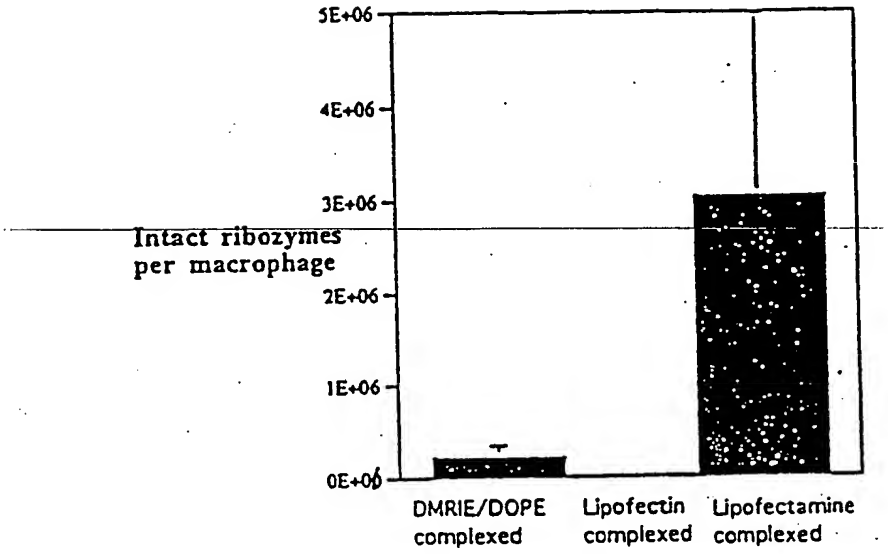
FIGURE 1



# FIGURE 3

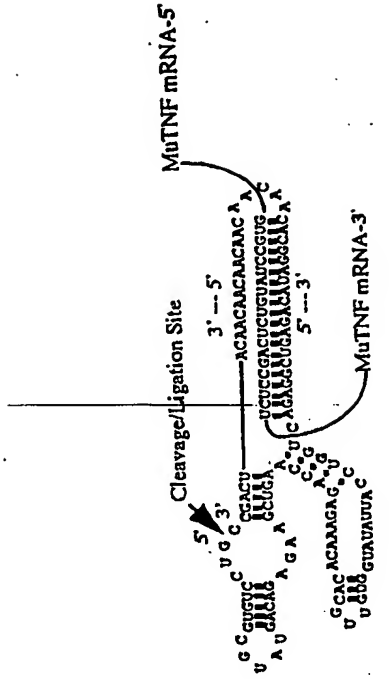


# FIGURE 5

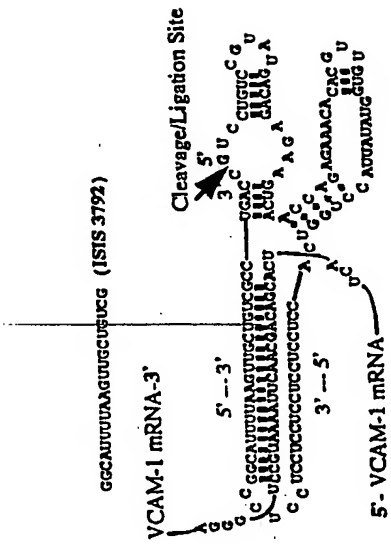



8 hours post administration

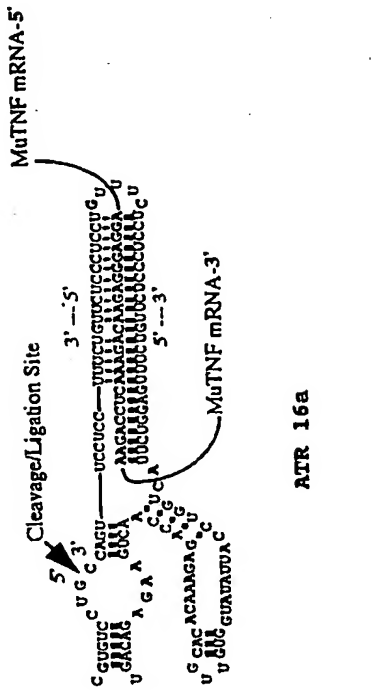
# FIGURE 7



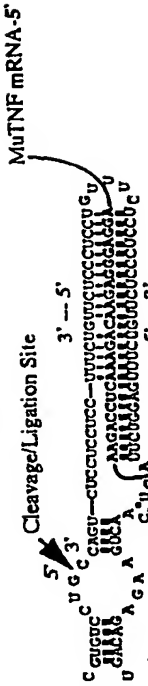

ALR 229




VALR 1

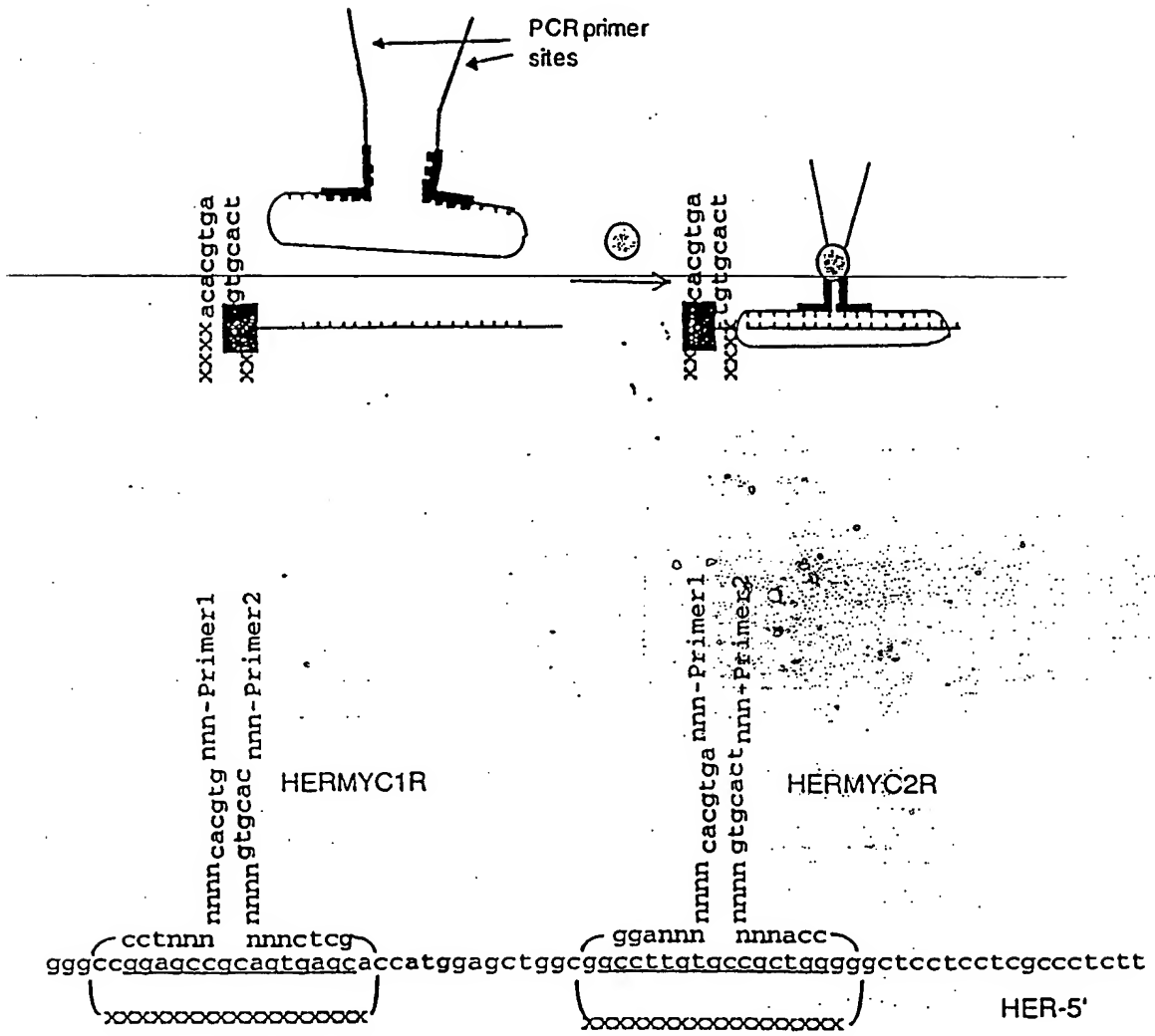



ATR 16a

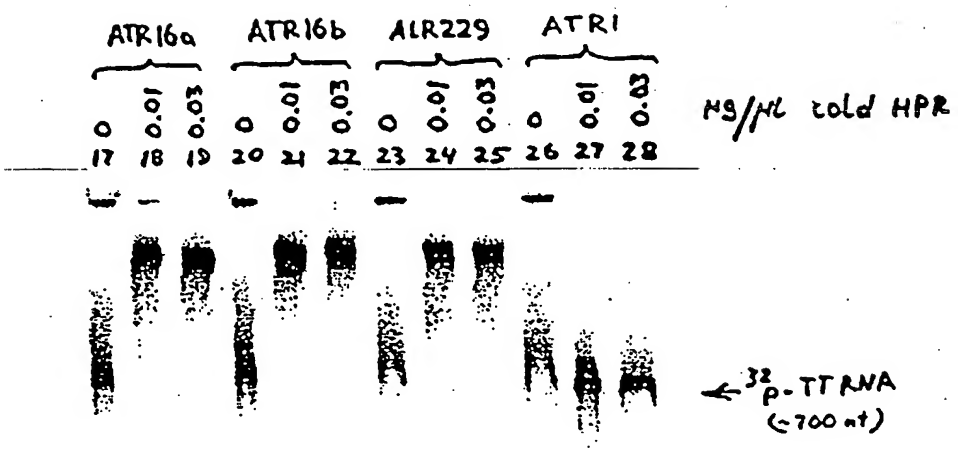



ATR 16b

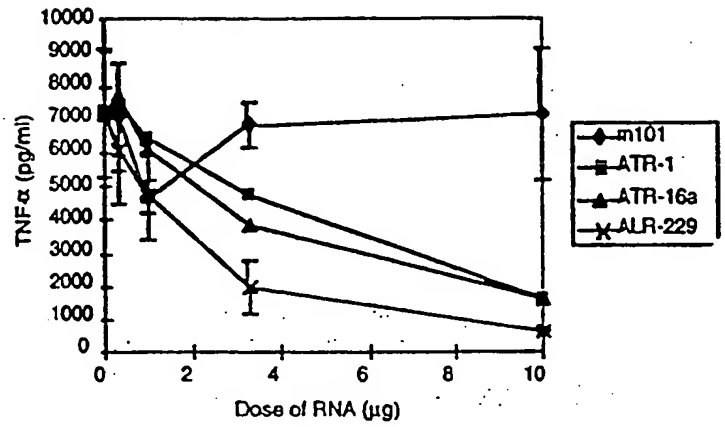
# FIGURE 9


PCR primer sites
xxxxacacgtga
xxxxgtgcact
xxxxacacgtga
xxxxgtgcact
nnn-Primer1
nnn-Primer2
HERMYC1R
nnn-Primer1
nnn-Primer2
HERMYC2R
nnnn cacgtg
nnnn gtgcac
nnnn cacgtga
nnnn gtgcact
cctnnn nnnctcg
ggannn nnnacc
gggccggagccgcagtgagcaccatggagctggcggccttgtgccgctgaggggctcctcctcgccctctt
xxxxxxxxxxxxxxxxx
xxxxxxxxxxxxxxxxx
HER-5'

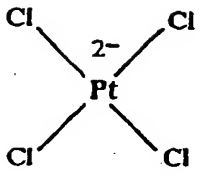
# FIGURE 11


ATR16a
ATR16b
ALR229
ATR1
0 0.01 0.03 0 0.01 0.03 0 0.01 0.03 0 0.01 0.03
mg/ml cold HPR
17 18 19 20 21 22 23 24 25 26 27 28
$^{32}$P-TT RNA
(~700 nt)

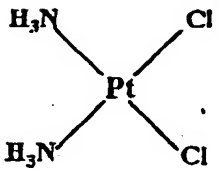
# FIGURE 13


10000
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
TNFα (pg/ml)
0
2
4
6
8
10
Dose of RNA (µg)
m101
ATR-1
ATR-16a
ALR-229

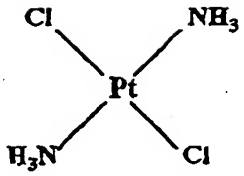
# FIGURE 15



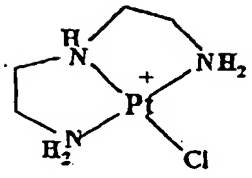

$[PtCl_4]^{2-}$



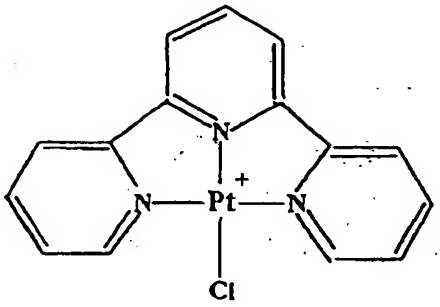

cis-$[(NH_3)_2PtCl_2]$




trans-$[(NH_3)_2PtCl_2]$

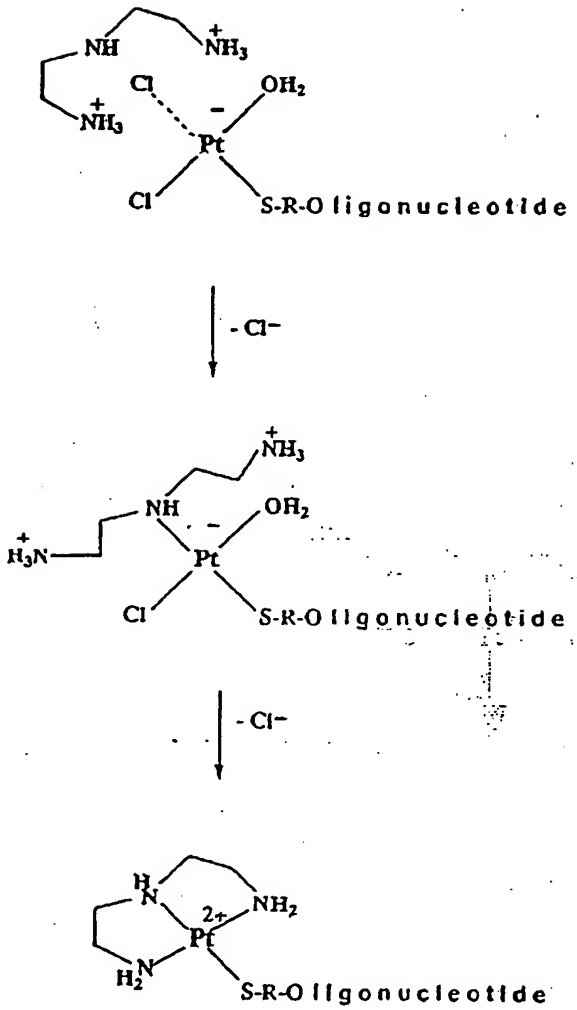



$[(dien)PtCl]^+$

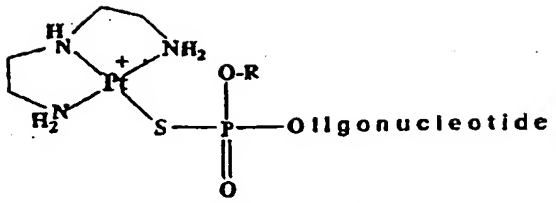



$[(terpy)PtCl]^+$

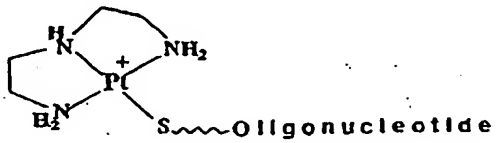
# FIGURE 17A


NH
NH3
Cl
OH2
NH3
Pt
Cl
S-R-Oligonucleotide
- Cl-
NH3
NH
OH2
H3N
Pt
Cl
S-R-Oligonucleotide
- Cl-
H2N
NH2
2+
Pt
H2N
S-R-Oligonucleotide

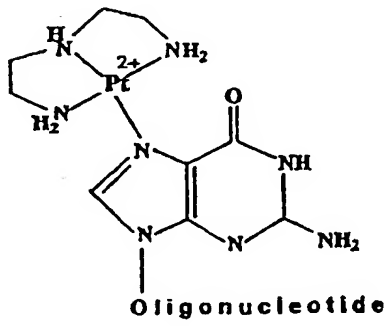
# FIGURE 17C




at internucleotide (R is an oligonucleotide fragment) and termini, 5-' or 3'- (R is H), phosphorothioates




at termini HS-$(CH_2)_n$-X groups




at guanine residue(s) in $G_n$ clusters (GG, GGG, GGGG etc.)

FIGURE 19

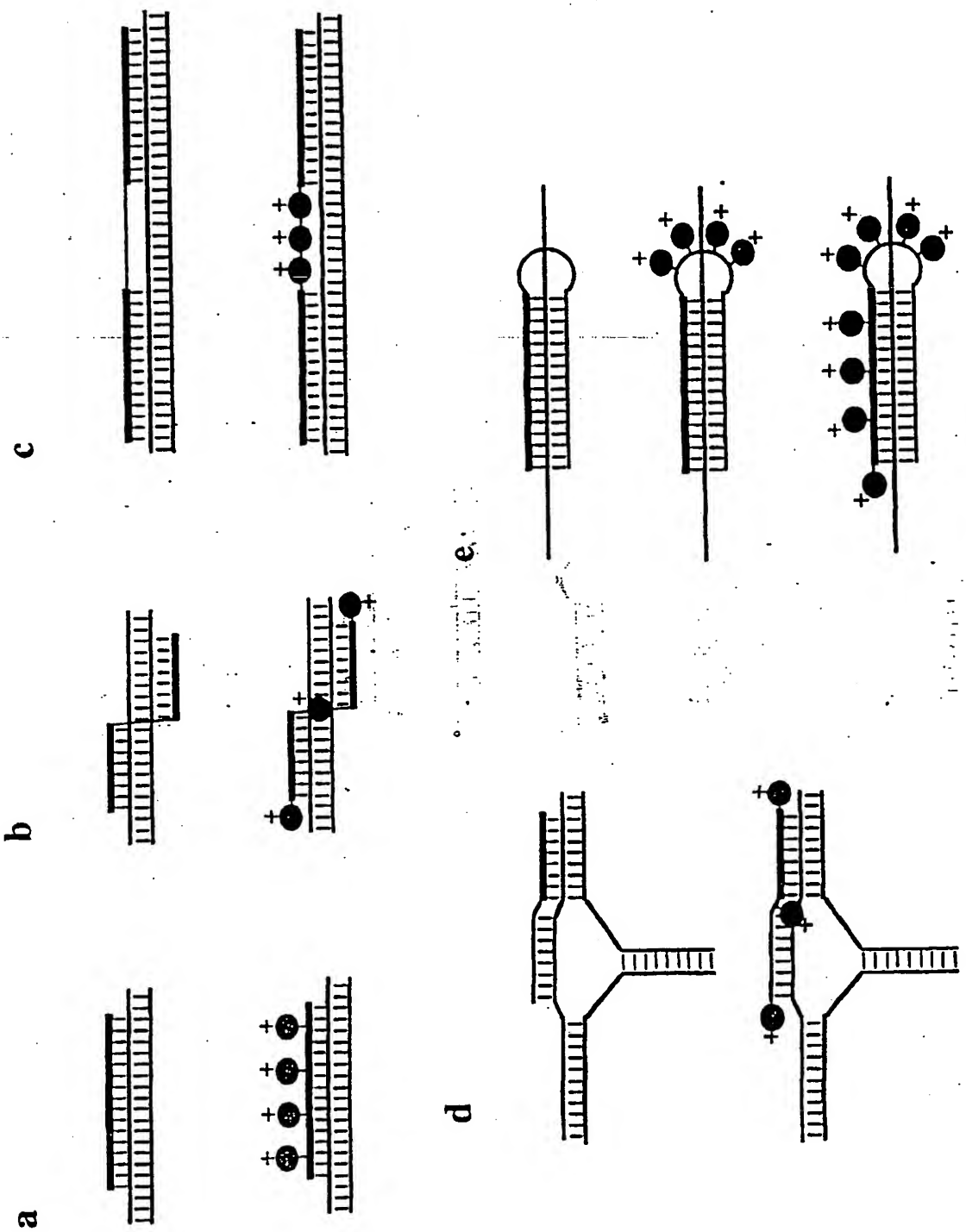
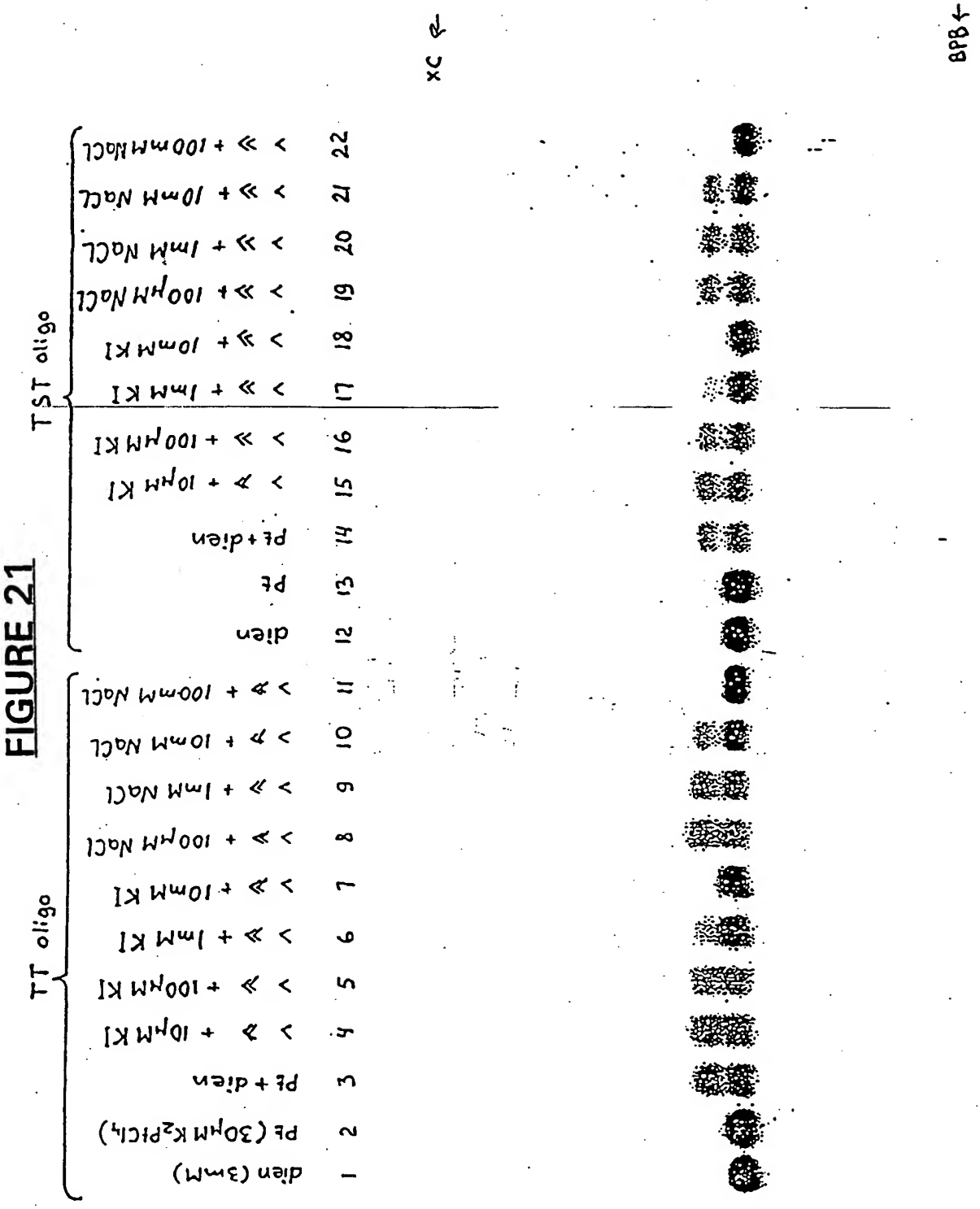
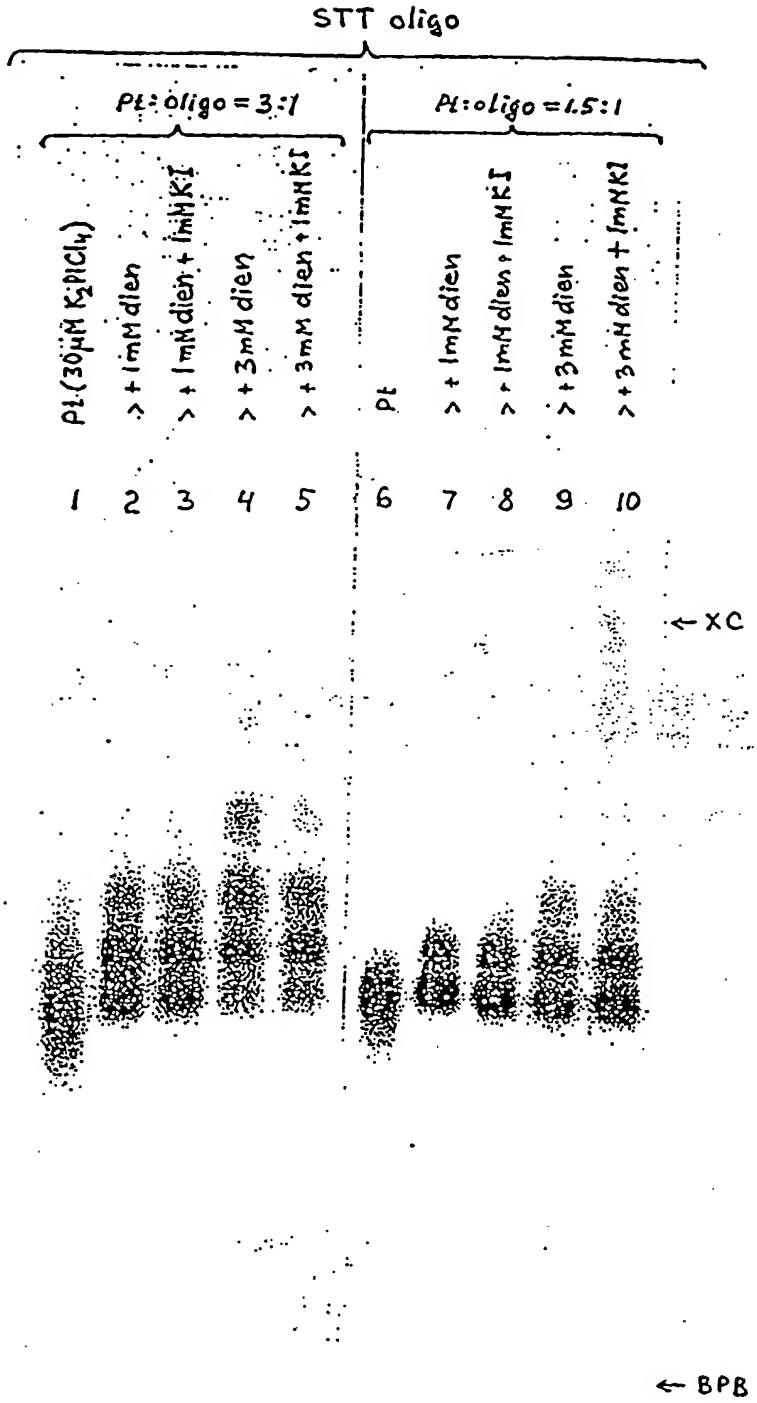


FIGURE 21


TT oligo
TST oligo
1 dien (3mM)
2 Pt (30μM K2PtCl4)
3 Pt + dien
4 > » + 10μM KI
5 > » + 100μM KI
6 > » + 1mM KI
7 > » + 10mM KI
8 > » + 100μM NaCl
9 > » + 1mM NaCl
10 > » + 10mM NaCl
11 > » + 100mM NaCl
12 dien
13 Pt
14 Pt + dien
15 > » + 10μM KI
16 > » + 100μM KI
17 > » + 1mM KI
18 > » + 10mM KI
19 > » + 100μM NaCl
20 > » + 1mM NaCl
21 > » + 10mM NaCl
22 > » + 100mM NaCl
XC
BPB

# FIGURE 23


STT oligo
Pt:oligo = 3:1
Pt:oligo = 1.5:1
Pt (30μM K2PtCl4)
Pt
+ 1mM dien
+ 1mM dien + 1mM KI
+ 3mM dien
+ 3mM dien + 1mM KI
1 2 3 4 5 6 7 8 9 10
← XC
← BPB

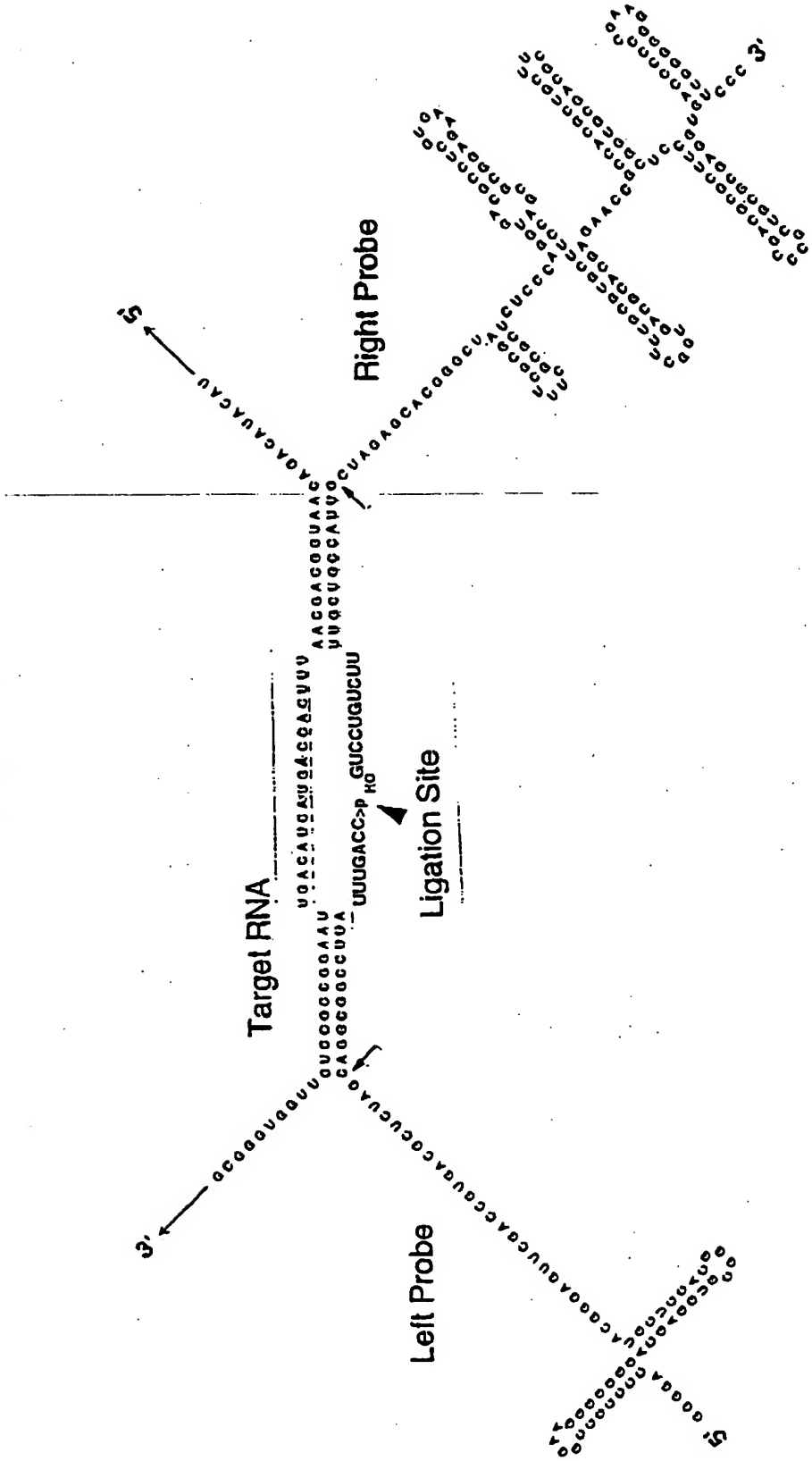



FIGURE 25

# FIGURE 27

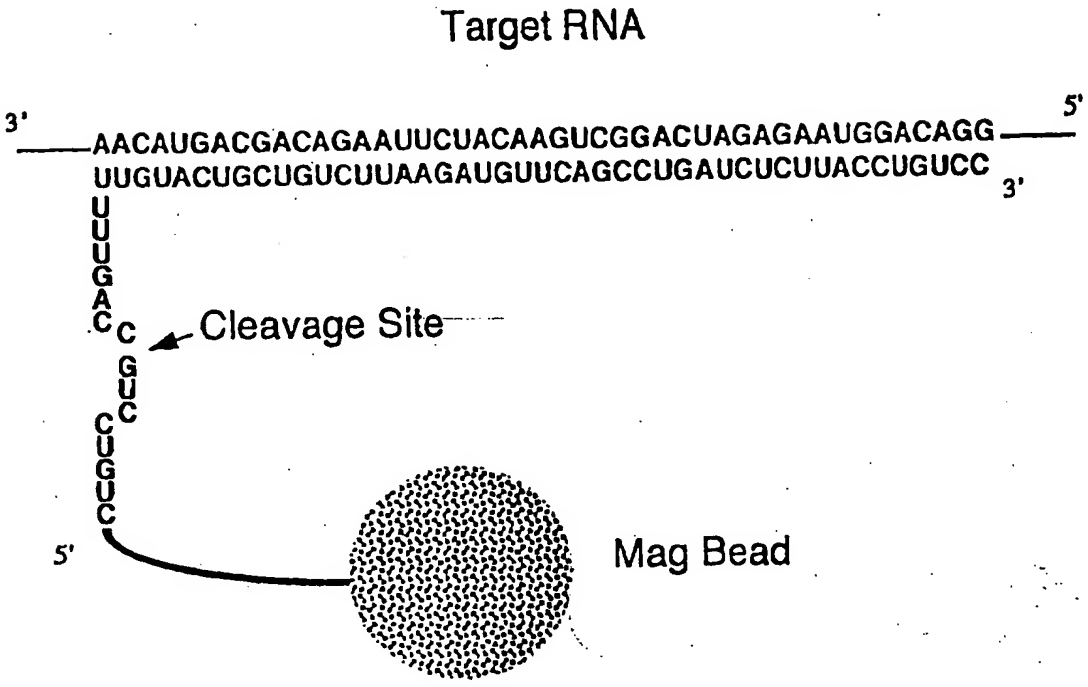
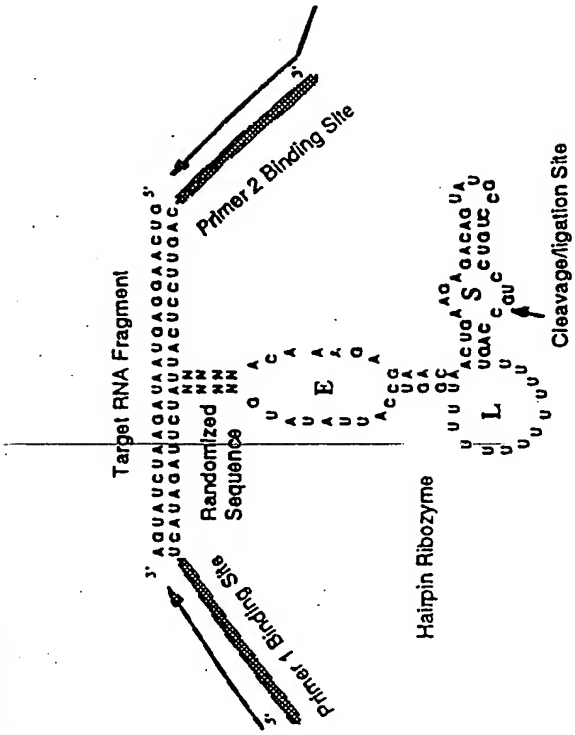

Target RNA
3'
AACAUGACGACAGAAUUCUACAAGUCGGACUAGAGAAUGGACAGG
5'
UUGUACUGCUGUCUUAAGAUGUUCAGCCUGAUCUCUUACCUGUCC
3'
UUUGAC
C
GUC
CUGUC
Cleavage Site
5'
Mag Bead

FIGURE 29


Target RNA Fragment
Primer 2 Binding Site
Randomized Sequence
Primer 1 Binding Site
Hairpin Ribozyme
Cleavage/ligation Site

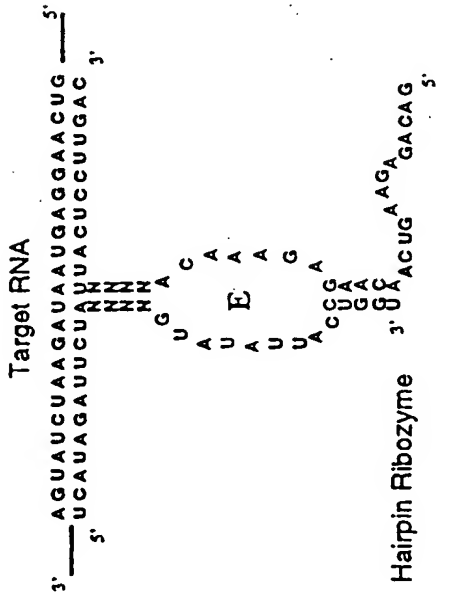
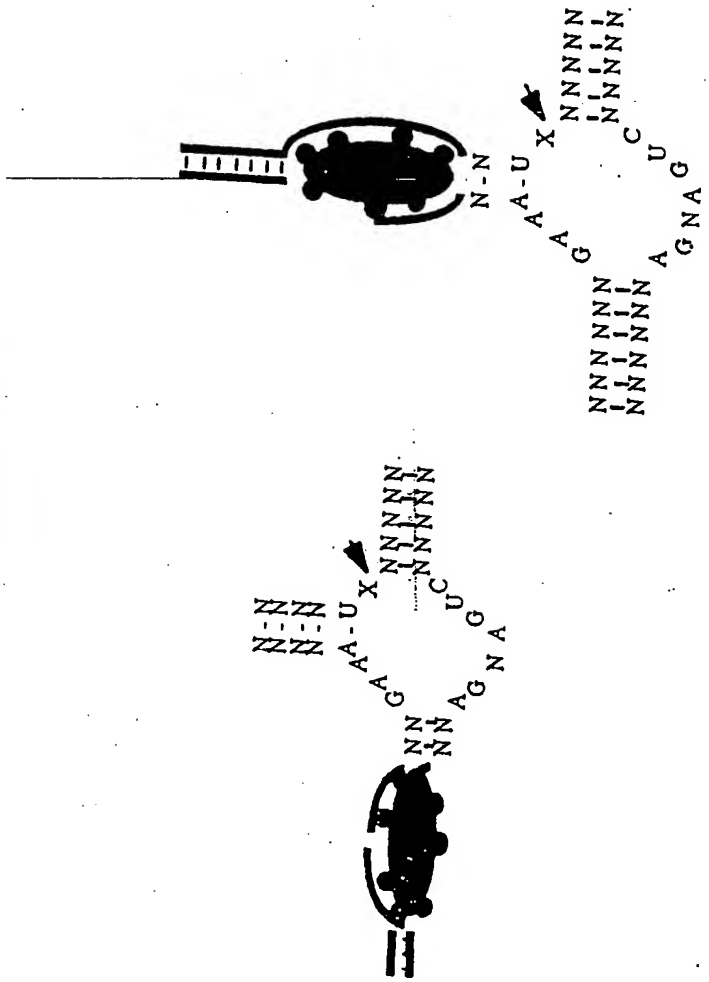

Target RNA
Hairpin Ribozyme

FIGURE 31



# INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/17268

A. CLASSIFICATION OF SUBJECT MATTER
IPC(6) : C07H 21/00, 21/02; C12Q 1/68
US CL : 435/6, 375; 536/ 24.3, 24.5, 25.3
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED
Minimum documentation searched (classification system followed by classification symbols)
U.S. : 435/6, 375; 536/ 24.3, 24.5, 25.3

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
APS, BIOSIS, CAPLUS, INPADOC, LIFESCI, MEDLINE, WPIDS
Search Terms: Oligonucleotide(s), Hoogsteen, foldback, clamp, lock(ing), ribozyme(s), platinum, polyamine(s), linker(s)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| X | US 5,473,060 A (GRYAZNOV et al.) 05 December 1995, see entire document, especially figures 1a and 1b. | 1, 7, 8, 18 |
| A | US 5,622,826 A (VARMA) 22 April 1997, see column 6, line 40 to col. 7, line 13, and Figures 1 and 7. | 19-22 |
| A | GIOVANNANGELI et al. Single-Stranded DNA as a Target for Triple-Helix Formation. Journal of the American Chemical Society. 1991, Vol. 113, No. 20, pages 7775-7777, see entire document. | 1-18 |

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

| | | | |
|---|---|---|---|
| * | Special categories of cited documents: | "T" | later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention |
| "A" | document defining the general state of the art which is not considered to be of particular relevance | "X" | document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone |
| "E" | earlier document published on or after the international filing date | "Y" | document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art |
| "L" | document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) | "&" | document member of the same patent family |
| "O" | document referring to an oral disclosure, use, exhibition or other means | | |
| "P" | document published prior to the international filing date but later than the priority date claimed | | |

Date of the actual completion of the international search
04 JANUARY 1999

Date of mailing of the international search report
27 JAN 1999

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Facsimile No. (703) 305-3230

Authorized officer
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Telephone No. (703) 308-0196

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